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PTO/SB/05 (12/97)

**UTILITY
PATENT
APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 19188PCA Total Pages 177

First Named Inventor or Application Identifier

M. A. Liu et al.

Express Mail Label No. EM358364529US

09/10/99
PTO
09/373803

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 148]
3. ☒ Drawing(s) (35 USC 113) [Total Sheets 27]
4. Oath or Declaration [Total Pages 2]
 - a. ☐ Newly executed (original or copy)
 - b. ☒ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 14 completed)
[Note Box 5 below]
 - i. ☐ **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked). The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

ACCOMPANYING APPLICATION PARTS

7. ☐ Assignment papers (cover sheet & document(s))
8. ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
9. ☒ Information Disclosure Statement (IDS)/PTO-1449 Power of Attorney
10. ☒ Preliminary Amendment
11. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
12. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
13. ☐ Other:

14. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No. 08 / 702,502

15. CORRESPONDENCE ADDRESS

☒ Customer Number or Bar Code Label

Customer No. 000210

(Insert Customer No. or Attach bar code here)

NAME	J. Mark Hand		
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(computer generated form No. U6 - Merck & Co., Inc., 12/97)

EXPRESS MAIL CERTIFICATE

DATE OF DEPOSIT September 10, 1999

EXPRESS MAIL NO. EM358,364529

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS EXPRESS MAIL "POST OFFICE TO ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, BOX PATENT APPLICATION, WASHINGTON, D.C. 20231.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
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Prior Application:

Examiner N. DEGEN

Art Unit 1636

Sir: This is a request for filing a ☒ Continuation ☐ Divisional

application, under 37 C.F.R. 1.53(b), of pending application Serial No. 08/702,502 filed on

March 3, 1997

of MARGARET A. LIU, JOHN W. SHIVER AND HELEN C. PERRY

(DATE)

(INVENTOR)

for COORDINATE IN VIVO GENE EXPRESSION

(TITLE OF INVENTION)

1. The enclosed comprises a true copy of prior application Serial No. 08/702,502, as filed on March 3, 1997

2. The filing fee is calculated below:

CLAIMS AS FILED IN THE PRIOR APPLICATION, LESS ANY CLAIMS CANCELED BY AMENDMENT BELOW

FOR	NUMBER FILED		NUMBER EXTRA		RATE	FEE
Total Claims	44	-	20	24	x	\$18 \$432
Independent Claims	14	-	3	11	x	\$78 \$858
*Multiple Dependent Claims					x	\$260
Basic Fee (minimum amount required)						\$760
						\$2050
						Total Fee

* Add this fee if application contains any multiple dependent claims, regardless of number.

3. The Assistant Commissioner is hereby authorized to charge any fees which may be required or to credit any overpayment to Account No. 13-2755.

4. ☒ Amend the specification by inserting before the first line the sentence:-- This is a ☒ continuation, ☐ division of application Serial No. 098/702,502
filed March 3, 19975. ☐ Cancel in this application original claims:5a. ☒ Preliminary amendment enclosed.6a. ☐ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.6b. ☐ New formal drawings are enclosed.7a. ☐ Priority of application Serial No. _____, filed _____,
in _____ is claimed under 35 U.S.C. 119.

(COUNTRY)

7b. ☐ The certified copy of the priority application has been filed in prior application Serial No. _____,
filed _____8. ☒ The prior application is assigned to MERCK & CO., INC. RAHWAY, N.J. 070659. ☒ The undersigned attorney or agent has:a. ☐ Power in the prior application as originally filed.b. ☒ Associate power, copy enclosed.

Respectfully,

EXPRESS MAIL CERTIFICATE

DATE OF DEPOSIT September 10, 1999

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By: J. Mark Hand

Attorney _____ for Applicant(s)

Registration No. 36,545

Telephone: (732) 594- 3905

Address: Merck & Co., Inc.

Patent Department

P. O. Box 2000 - RY60-30

Rahway, N.J. 07065-0907

Date: September 10, 1999

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Liu et al.		
Serial No.:	To be assigned	Case No.:	19188PCA
Art Unit:	1805		
Filed:	Submitted herewith		
Examiner:	Nancy J. Degan		
For:	COORDINATE IN VIVO EXPRESSION		

Assistant Commissioner of Patents
Washington, D.C. 20231

EXPRESS MAILING CERTIFICATE, 37 C.F.R. 1.10
DATE OF DEPOSIT September 10, 1999
EXPRESS MAILING NO EM 358364529US

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PRELIMINARY AMENDMENT, 37 C.F.R. 1.115

ASSISTANT COMMISSIONER OF PATENTS
BOX PATENT APPLICATIONS
WASHINGTON, DC 20231

MAILED BY Devo Samant
DATE Sept. 16, 1999

Sir:

Preliminary to the examination of this concurrently submitted Rule 53(b) continuation application, please enter the following amendments and consider the following remarks.

An Office Action was issued on March 12, 1999 in the parent case, U.S. Application Serial No. 08/702,502. Therefore, a petition to extend time for responding to the Office Action is being submitted separately in the '502 application in order to keep the '502 application pending through at least Monday, September 13, 1999.

In the event any additional fee is required for entry of this Amendment or any paper related to this Amendment, please charge the fee to Deposit Account No. 13-2755, as a large entity. Please credit any overpayment or charge any fee deficiency to Deposit Account No. 13-2755.

IN THE CLAIMS:

Please amend claims 1, 2, 18, 22, 24, 35, 39, 41, 42 and 44, as follows:

1(Amended). A polynucleotide which, upon in vivo introduction into a mammalian cell, is non-replicating and induces the co-expression in the cell of at least two gene products, comprising:

a first transcriptional promoter which operates in eukaryotic cells upstream from, and in transcriptional control of, a first cistron;

a second cistron downstream from the first cistron, under transcriptional control either of the first transcriptional promoter or under control of a second transcriptional promoter;

optionally, a third cistron downstream from the second cistron, under transcriptional control either of the first transcriptional promoter or under control of the second transcriptional promoter, or under control of a third transcriptional promoter; and

a transcriptional terminator following each of the first, second and third cistron, unless followed by another cistron lacking its own transcriptional promoter.

2(Amended). The polynucleotide of Claim 1 wherein the first cistron encodes at least one immunogenic epitope of a pathogen [or a cancer associated pathogen].

18(Amended). A polynucleotide which comprises contiguous nucleic acid sequences which cannot replicate in eukaryotic cells in vivo but which are capable of being expressed to produce a gene product upon introduction of the polynucleotide into eukaryotic tissues *in vivo*, wherein the gene product either acts as an immunostimulant or as an antigen capable of generating an immune response, wherein the nucleic acid sequences encode:

- a) a spliced REV gene;
- b) a spliced human immunodeficiency virus (HIV) immunogenic epitope; and,
- c) optionally, a cytokine or a T-cell recognition element.

22(Amended). A polynucleotide comprising a first gene encoding an HIV gag, gag-protease, or env immunogenic epitope, the gene containing a REV responsive element (RRE) or having been modified to contain an RRE, the gene being operatively linked with a transcriptional promoter suitable for gene expression in a mammal, the gene being linked with an internal ribosome entry site (IRES), and the IRES being linked with a gene encoding a REV gene product, wherein said polynucleotide is non-replicating in eukaryotic cells in vivo.

24(Amended) . A polynucleotide which is non-replicating in eukaryotic cells in vivo and induces anti-HIV neutralizing antibody, HIV specific T-cell immune responses, or protective immune responses upon introduction into vertebrate tissue, including human tissue *in vivo*, wherein the polynucleotide comprises a gene encoding a gene product selected from HIV gag, HIV gag-protease, and HIV env, the gene containing a REV responsive element (RRE), the gene being operatively linked with a transcriptional promoter suitable for gene expression in a mammal, the gene being linked with an internal ribosome entry site (IRES), and the IRES being linked with a second gene, the second gene encoding a REV gene product.

35(Amended). A polynucleotide which is non-replicating in eukaryotic cells in vivo, comprising:

- a) an eukaryotic transcriptional promoter;
- b) an open reading frame 3' to the transcriptional promoter encoding an immunogenic HIV epitope wherein the open reading frame has a splice donor sequence at the 5'-side of the open reading frame, a REV responsive element anywhere within the open reading frame, and a stop codon encoding the termination of translation of the open reading frame;
- c) an internal ribosome entry site (IRES) 3' to the translation stop codon of the open reading frame;
- d) an open reading frame encoding a spliced HIV REV gene at the 3' end of which is a translation stop codon;

- e) optionally, 3' to the REV translation stop codon, a second IRES, followed by an open reading frame encoding immunomodulatory or immunostimulatory genes, the genes being selected from GM-CSF, IL-12, interferon, and a B7 protein;
- f) a transcription-termination signal following the last open reading frames.

39(Amended). A polynucleotide which is non-replicating in eukaryotic cells in vivo, [comprises] comprising sequences encoding:

- a) an eukaryotic transcription initiation signal;
- b) an HIV gene open reading frame (ORF) preceded by an heterologous leader sequence such that expression of the HIV gene ORF does not depend on availability of the HIV REV gene product;
- c) a sequence which operates as an internal ribosome entry site (IRES) 3' to the translation stop codon of the HIV ORF;
- d) a sequence encoding an ORF of a T-cell costimulatory element 3' to the IRES; and
- e) a transcription termination signal 3' to the translation stop codon of the T-cell costimulatory element.

41(Amended). A polynucleotide which is non-replicating in eukaryotic cells in vivo, [comprises] comprising sequences encoding:

- a) an eukaryotic transcription initiation signal;
- b) an HIV gene open reading frame (ORF) preceded by an heterologous leader sequence such that expression of the HIV gene ORF does not depend on availability of the HIV REV gene product;
- c) a sequence which operates as an internal ribosome entry site (IRES) 3' to the translation stop codon of the HIV ORF;
- d) an HIV gene open reading frame (ORF) preceded by an heterologous leader sequence such that expression of the HIV gene ORF does not depend on availability of the HIV REV gene product; and
- e) a transcription termination signal 3' to the translation stop codon of the HIV gene ORF.

42(Amended). A composition comprising multiple expression constructs of claim 1, each of which is capable of inducing expression in mammalian tissue of more than a single cistron encoding antigens related to disease causing pathogens or tumors.

44(Amended). A polynucleotide construct which is non-replicating in eukaryotic cells in vivo, having the elements shown in figure 2, wherein each of the first, second and third cistrons shown in the figure encode a combination of any two to three of the following:

- 1) tPA-gp120MN;
- 2) gp160^{IIIB}/IRES/REV^{IIIB};
- 3) gp160^{IIIB};
- 4) REV^{IIIB};
- 5) tat/REV/gp160;
- 6) REV/gp160;
- 7) gp160MN;
- 8) gp160 from clinically relevant primary HIV isolates;
- 9) nef, using the gene from clinically relevant strains;
- 10) gag^{IIIB};
- 11) tPA-gp120^{IIIB};
- 12) gp160 with structural mutations including V3 loop substitutions from clinically relevant strains of HIV; several mutations on several constructs such as variable loop removal, Asn mutations to remove steric carbohydrate obstacles to structural, neutralizing antibody epitopes; and CD4 binding site knockout mutants;
- 13) gp41 with provision of appropriate leader sequences, as in the tPA signal peptide leader sequence;
- 14) gag: similar to construct from #5 above, using the gene from clinically relevant strains;
- 15) rev: for gp160 and gag dicistronics;
- 16) B7 coding sequences;
- 17) GM-CSF sequences;
- 18) Interleukin sequences;
- 19) Tumor associated antigens;

20) Genes encoding antigens expressed by pathogens other than HIV, such as, but not limited to, influenza virus nucleoprotein, hemagglutinin, matrix, neuraminidase, and other antigenic proteins; herpes simplex virus genes; human papillomavirus genes; tuberculosis antigens; hepatitis A, B, or C virus antigens; and combinations of these and other antigens to form at least dicistronic constructs which may be combined with multiple other polycistronic constructs to provide a cocktail composition capable of raising immune responses against all of the represented pathogens or tumor antigens;

wherein the segments A and B of figure 2 are internal ribosome entry sites or a combination of transcription termination sequences terminating the transcription of the upstream cistron and transcriptional promoter sequences, initiating the transcription of downstream cistron.

IN THE ABSTRACT OF THE DISCLOSURE:

At a new page 148, after page 147, which ends with original claim 44, please add the following Abstract of the Disclosure:

--

ABSTRACT OF THE DISCLOSURE

Nucleic acids, including DNA constructs and RNA transcripts, capable of coordinate expression of two or three cistrons upon direct introduction into animal tissues, are presented. Bi- and tri-cistronic polynucleotides of this invention include those encoding and co-expressing HIV gene products, genes encoding antigens unrelated to HIV, and immunostimulatory gene products, including but not limited to GM-CSF, interleukins, interferon and members of the B7 family of proteins which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression in vivo of any two or more genes in a single cell. --.

REMARKS

An Office Action was issued on March 12, 1999 in the parent case, U.S. Application Serial No. 08/702,502. Applicants choose to pursue further prosecution of the '502 subject matter through entry of the Rule 53(b) continuation application and this Preliminary Amendment. Therefore, a petition to extend time for responding to the '502 application Office Action is being submitted separately in the '502 application file in order to keep the '502 application pending through at least Monday, September 13, 1999.

The specification was amended to add an Abstract of the Disclosure as new page 148. No new matter is added by introduction of the Abstract.

Claims 1, 18, 22, 24, 35, 39, 41, 42 and 44 were amended to more particularly point out and distinctly claim the subject matter Applicants regard as their invention.

Claim 2 was amended herein to delete recitation of cancer vaccine and applications.

Applicants reserve the right to pursue canceled subject matter in a later continuing application.

No new matter is added by these claim amendments.

The claims are now in better form for examination and allowance. Early action to that end is earnestly solicited. The Examiner is invited to contact the undersigned attorney if clarification is required on any aspect of this response, or if any of the claims are considered to require further amendment to be placed in condition for allowance.

Respectfully submitted,



J. Mark Hand

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Attorney for Applicants
Merck & Co., Inc.
P.O. Box 2000
Rahway, NJ 07065-0907
(732) 594-3905

Date: SEPTEMBER 10, 1999

TITLE OF THE INVENTION
COORDINATE IN VIVO GENE EXPRESSION

CROSS-RELATED TO OTHER APPLICATIONS

5 This is a continuation-in-part of U. S. Serial Number
08/207,526, filed March 7, 1994, now pending.

BACKGROUND OF THE INVENTION

1. Field of the Invention

10 A method for coordinate expression in a single cell, in vivo, of exogenous genes via introduction into the tissue of a vertebrate of polycistronic polynucleotide constructs is described. The method results in production of immune responses against the products
15 produced as a result of expression of the exogenous genes. The method and polynucleotide constructs of this invention may be used in a vertebrate to generate immune responses against antigenic epitopes expressed by a single cell. The coordinate expression results in improved expression of gene products which may be otherwise poorly
20 expressed. It also results in improved cellular immune responses due to provision of T-cell stimulatory signals by the same cell expressing T-cell antigens. Polynucleotide constructs encoding human immunodeficiency virus (HIV) antigens exemplify one embodiment of the method.

25 2. Background of the Invention

 A major challenge to the development of vaccines against viruses, particularly viruses with a high rate of mutation such as HIV, against which elicitation of neutralizing and protective immune
30 responses is desirable, is the diversity of the viral envelope proteins among different viral isolates or strains. Because cytotoxic T-lymphocytes (CTLs) in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins and may be important in the immune response against viruses, efforts have

been directed towards the development of CTL vaccines that elicit heterologous protection against different viral strains.

CD8⁺ CTLs kill virally-infected cells when their T cell receptors recognize viral peptides associated with MHC class I molecules. These peptides are derived from endogenously synthesized viral proteins. Thus, by recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. Peptides capable of associating with MHC class I for CTL recognition originate from proteins that are present in or pass through the cytoplasm or endoplasmic reticulum. Exogenous proteins which enter the endosomal processing pathway (as in the case of antigens presented by MHC class II molecules) are not usually effective in generating CD8⁺ CTL responses.

Efforts to generate CTL responses have used replicating vectors to produce the protein antigen within the cell or have introduced peptides into the cytosol. These approaches have limitations that may limit their utility as vaccines. Retroviral vectors have restrictions on the size and structure of polypeptides that can be expressed as fusion proteins while maintaining the ability of the recombinant virus to replicate. Further, the effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against the vectors themselves. Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans [R.R. Redfield *et al.*, New Engl. J. Med. **316**, 673 (1987); L. Mascola *et al.*, Arch. Intern. Med. **149**, 1569 (1989)]. Furthermore, the selection of peptide epitopes to be presented is dependent upon the structure of an individual's MHC antigens; thus, peptide vaccines may have limited effectiveness due to the diversity of MHC haplotypes in outbred populations.

Benvenisty, N., and Reshef, L. [PNAS **83**, 9551-9555, (1986)] showed that CaCl₂-precipitated DNA introduced into mice intraperitoneally (i.p.), intravenously (i.v.) or intramuscularly (i.m.) could be expressed. Intramuscular injection of DNA expression vectors in mice results in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA [J.A. Wolff *et al.*, Science **247**,

1465 (1990); G. Ascadi *et al.*, *Nature* **352**, 815 (1991)]. The plasmids were maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats. The technique of using nucleic acids as therapeutic agents was reported in WO90/11092 (4 October 1990), in which naked polynucleotides were used to vaccinate vertebrates.

It is not necessary for the success of the method that immunization be intramuscular. Thus, Tang et al., [*Nature*, **356**, 152-154 (1992)] disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice. Furth et al., [*Anal. Biochem.* **205**, 365-368, (1992)] showed that a jet injector could be used to transfect skin, muscle, fat, and mammary tissues of living animals. Methods for introducing nucleic acids was recently reviewed by Friedman, T., [*Science*, **244**, 1275-1281 (1989)]. Robinson et al., [Abstracts of Papers Presented at the 1992 meeting on Modern Approaches to New Vaccines, Including Prevention of AIDS, Cold Spring Harbor, p92] reported that i.m., i.p., and i.v. administration of avian influenza DNA into chickens provided protection against lethal challenge. However, Robinson et al. did not disclose which avian influenza virus genes were used. In addition, only H7 specific immune responses were alleged; the induction of cross-strain protection was not discussed. Intravenous injection of a DNA:cationic liposome complex in mice was shown by Zhu et al., [*Science* **261**:209-211 (9 July 1993); see also WO93/24640, 9 Dec. 1993] to result in systemic expression of a cloned transgene. Recently, Ulmer et al., [*Science* **259**:1745-1749, (1993)] reported on the heterologous protection against influenza virus infection by injection of DNA encoding influenza virus proteins.

The need for specific therapeutic and prophylactic agents capable of eliciting desired immune responses against pathogens and tumor antigens is achieved by the instant invention. Of particular importance in this therapeutic approach is the ability to induce T-cell

immune responses which can prevent infections or disease caused by virus strains which are heterologous to the strain from which the antigen gene was obtained. This is of significance with HIV, since HIV mutates rapidly, and because many virulent isolates have been identified [see, for example, LaRosa et al., Science 249:932-935 (1990), identifying 245 separate HIV isolates].

In response to this diversity, researchers have attempted to generate CTLs by peptide immunization. Thus, Takahashi et al., [Science 255:333-336 (1992)] reported on the induction of broadly cross-reactive cytotoxic T cells recognizing an HIV envelope (gp160) determinant. They recognized the difficulty in achieving a truly cross-reactive CTL response and suggested that there is a dichotomy between the priming or restimulation of T cells, which is very stringent, and the elicitation of effector function, including cytotoxicity, from already stimulated CTLs.

Wang et al., [P.N.A.S. USA 90:4156-4160 (May, 1993)] reported on elicitation of immune responses in mice against HIV by intramuscular inoculation with a cloned, genomic (unspliced) HIV gene. The level of immune response achieved was low, and the system utilized portions of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter and portions of the simian virus 40 (SV40) promoter and terminator. SV40 is known to transform cells, possibly through integration into host cellular DNA. Therefore, unlike the system described herein, the system described by Wang et al. may be inappropriate for administration to humans. In addition, the DNA construct of Wang et al. contains an essentially genomic piece of HIV encoding contiguous Tat/REV-gp160-Tat/REV coding sequences (Figure 1). As is described in detail below, this is a suboptimal system for obtaining high-level expression of the gp160. One drawback is that the expression of Tat has been recognized to play a contributory role in the progression of Kaposi's Sarcoma, [Y.N. Vaishav and F.W. Wong-Staal, An. Rev. Biochem. (1991)].

WO 93/17706 describes a method for vaccinating an animal against a virus, wherein carrier particles were coated with a gene

construct and the coated particles are accelerated into cells of an animal. In regard to HIV, essentially the entire genome, minus the long terminal repeats, was proposed to be used. That method may represent a substantial risk for recipients. Constructs of HIV should, in general, contain less than about 50% of the HIV genome to ensure safety of the vaccine. Thus, a number of problems remain if a useful human HIV vaccine is to emerge from the gene-delivery technology.

The instant invention uses known methods for introducing polynucleotides into living tissue to induce expression of proteins. This invention provides a immunogen for introducing HIV and other proteins into the antigen processing pathway to efficiently generate HIV-specific CTLs and antibodies. The pharmaceutical is effective as a vaccine to induce both cellular and humoral anti-HIV and HIV neutralizing immune responses. The instant invention addresses some of the problems by providing polynucleotide immunogens which, when introduced into an animal, direct the efficient expression of HIV proteins and epitopes without the attendant risks associated with those methods. The immune responses generated are effective at recognizing HIV, at inhibiting replication of HIV, at identifying and killing cells infected with HIV, and are cross-reactive against many HIV strains. Therefore, this invention provides a useful immunogen against HIV. The invention also provides polynucleotide constructs which enable the co-expression, in vivo, of more than one gene-product in a single cell. This is demonstrated with an HIV gene expression system in which the expression of a first gene is dependent on the co-expression in the same cell of a second gene product. By virtue of the success of achieving this co-expression in vivo, it is now predictable that this type of polynucleotide construct may be applied to co-expression in vivo of many combinations of gene products, including but not limited to viral antigens other than HIV related antigens, carcinoma-associated antigens, and immunomodulatory or immunostimulatory gene products.

SUMMARY OF THE INVENTION

Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing coordinate expression of two to three cistrons upon direct introduction into animal tissues, are presented. In one embodiment, coordinate expression of two cistrons encoding HIV proteins and elicitation of HIV specific immune responses against more than one gene products is demonstrated. Cytotoxic T lymphocytes (CTLs) specific for viral antigens which respond to different strains of human immunodeficiency virus (HIV), and antibodies which are generally strain-specific are generated. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as in the case of virus infection. To generate a viral antigen for presentation to the immune system, without the limitations of direct peptide delivery or the use of viral vectors, polynucleotides encoding HIV proteins are directly introduced into tissues of vertebrates in vivo, the polynucleotides are taken up by cells within the tissue, and the encoded proteins produced and processed for presentation to the immune system. In mice, this resulted in the generation of HIV-specific CTLs and antibodies. Similar results are achieved in primates. These results are achieved with bi- or tri-cistronic nucleic acid polynucleotides encoding and co-expressing HIV gene products, immunostimulatory gene products including but not limited to GM-CSF, interleukins, interferon and B7 proteins, which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression in vivo of any two or three genes. Thus, various embodiments of this invention include coordinate expression in vivo of viral antigens and immunostimulatory gene products as well as coordinate expression of tumor antigens and immunostimulatory genes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. A schematic representation of the HIV genome.

Fig. 2. A schematic representation of a polynucleotide construct of this invention capable of inducing the co-ordinate expression in vivo in a single cell of up to three gene products encoded

by each of three cistrons (I, II, and III). The segments A and B represent control sequences including transcription termination signals and promoters or internal ribosome entry sites (IRES).

5 Fig. 3. Detailed schematic of an HIV env polynucleotide immunogen construct comprising the CMV-intA transcription promoter, a 5'-splice donor, HIV gp160 (showing gp120, gp41, and the REV-responsive element, RRE), an internal ribosome entry site (IRES), the REV cistron, the BGH transcription terminator, and the neomycin resistance marker which is driven by a prokaryotic transcription promoter..

10 Fig. 4. Detailed schematic of dicistronic HIV env and gag polynucleotide immunogen constructs showing specific regulatory elements.

15 Fig. 5. Western blot analysis of gp160 expression induced by HIV polynucleotide immunogens. This result rigorously shows the coexpression in a single cell of more than one gene product from a single polynucleotide construct: A polynucleotide encoding gp160 alone (see panel B, fourth lane from the left) expresses no detectable gp160, but with REV added in trans (by cotransfection of a construct encoding only REV), there is good gp160 expression (panel A, fourth lane from the left). A genomic tat/REV/env construct expresses only low levels of gp160, whether or not REV is provided in trans (panels A and B, third lane). However, a dicistronic gp160/IRES/REV construct heavily expresses gp160 (panels A and B, fifth lane from the left). The best expression, is obtained in a dicistronic construct encoding gp160/IRES/REV, with a splice donor (SD) provided 5' to the gp160 coding sequence (panels A and B, right hand lane). Because no additional expression is achieved when additional REV is provided in trans (panel A right hand lane), the system is not limited by the level of REV being expressed.

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25

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Fig. 6. V1J Sequence.

Fig. 7. V1Jneo Sequence.

Fig. 8. CMVintABGH Sequence.

Fig. 9. Cytotoxic T lymphocytes generated in rhesus monkeys in response to V1J-SIV-p28 polynucleotide construct vaccination (REV independent). This SIV p28 is equivalent to p24 gag of HIV. Thus, CTLs specific to a group specific antigen are inducible using a gag encoding polynucleotide construct.

Fig.10. Cytotoxic T lymphocytes generated in response to Vaccinia-SIVp28 nucleic acid vaccination. This demonstrates that similar CTLs are induced by a gag encoding polynucleotide (figure 9) as compared with a replicating antigen (vaccinia) expressing the same antigen [see Shen, L., et al., Science 252:440-443, 1991].

Fig.11. Sequence of the Vector V1R.

Fig.12. Antibodies induced by V1Jns-tPA-gp120, 200 µg/mouse per round, 2 rounds.

Fig.13. Neutralization of HIV-1 (MN) virus by sera from V1Jns-tPA-gp120 (MN) DNA vaccinated African Green Monkeys. Panels a and B show the reduction in p24 gag protein production for C8166 cells infected with HIV-1 (MN) following exposure to the indicated dilutions of sera from V1Jns-tPA-gp120 DNA vaccinated monkeys. Data was obtained after 10 days in tissue culture following virus inoculation (TCID₅₀ per sample).

Fig.14 T cells from V1Jns-tPA-gp120 vaccinated mice exhibiting long-term, antigen-specific T lymphocyte memory responses.

Immunized mice received 1.6 mcg of vaccine DNA twice, six months prior to sacrifice. Splenic T cells were cultured in vitro with recombinant gp120 protein at 5 mcg/mL. Proliferation of gp120-specific T cells. A stimulation index (SI; incorporated ³H-thymidine for gp120 treated T cells:T cells that did not receive antigen).

Fig. 15. Type 1 T helper (TH1) lymphocyte cytokine secretion by T cells from V1Jns-tPA-gp120 DNA vaccinated mice. Cell culture supernatants from the samples shown in Figure 13 were assayed for gamma-interferon and interleukin 4 (IL-4) secretion following

treatment with rgp120. Immune mice secreted large amounts of gamma-interferon and very low amounts of IL-4 indicated that TH1-like responses were induced by this vaccine. Control mice showed very low amounts of interferon secretion while the IL-4 levels indicated are background levels.

Fig. 15 Anti-gp120 cytotoxic T lymphocyte (CTL) activities in V1Jns-tPA-gp120 DNA vaccinated mice. Two mice (2006 and 2008) showed MHC I restricted CTL activities specific to a gp120 peptide (P18) following gp120 DNA vaccinations. No activities were observed for these mice in the absence of P18 or by a control mouse which had not been previously vaccinated.

Fig. 16. Anti-gp160 CTL activities by rhesus monkeys vaccinated with V1Jns-gp160/IRES/rev and V1Jns-tPA-gp120 DNA vaccines. T lymphocyte cultures from all four monkeys receiving these vaccines showed MHC I restricted killing of autologous target cells that had been treated with vaccinia-gp160. No CTL activity was observed in four control rhesus that had been immunized with 'blank' DNA vaccine (V1Jns without a gene insert).

DETAILED DESCRIPTION OF THE INVENTION

Nucleic acids, including DNA constructs and RNA transcripts, capable of inducing coordinate expression of two to three cistrons upon direct introduction into animal tissues, are presented. In one embodiment, coordinate expression of two cistrons encoding HIV proteins and elicitation of HIV specific immune responses against more than one gene products is demonstrated. Cytotoxic T lymphocytes (CTLs) specific for viral antigens which respond to different strains of human immunodeficiency virus (HIV), and antibodies which are generally strain-specific are generated. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as in the case of virus infection. To generate a viral antigen for presentation to the immune system, without the limitations of direct peptide delivery or the use of viral vectors, polynucleotides encoding HIV proteins are directly introduced into tissues of vertebrates in vivo, the

polynucleotides are taken up by cells within the tissue, and the encoded proteins produced and processed for presentation to the immune system. In mice, this resulted in the generation of HIV-specific CTLs and antibodies. Similar results are achieved in primates. These results are achieved with bi- or tri-cistronic nucleic acid polynucleotides encoding and co-expressing HIV gene products, immunostimulatory gene products including but not limited to GM-CSF, interleukins, interferon and B7 proteins, which act as T-cell costimulatory elements. The methods and polynucleotides of this invention are generally applicable to co-ordinate expression in vivo of any two or three genes. Thus, various embodiments of this invention include coordinate expression in vivo of viral antigens and immunostimulatory gene products as well as coordinate expression of tumor antigens and immunostimulatory genes.

This invention provides polynucleotides which, when directly introduced into a vertebrate in vivo, including mammals such as primates and humans, induces the expression of encoded proteins within the animal.

As used herein, a polynucleotide is a nucleic acid which contains essential regulatory elements such that upon introduction into a living vertebrate cell, is able to direct the cellular machinery to produce translation products encoded by the genes comprising the polynucleotide.

In one embodiment of the invention, the polynucleotide is a polydeoxyribonucleic acid comprising HIV genes operatively linked to a transcriptional promoter. In another embodiment of the invention, the polynucleotide vaccine comprises polyribonucleic acid encoding HIV genes which are amenable to translation by the eukaryotic cellular machinery (ribosomes, tRNAs, and other translation factors). Where the protein encoded by the polynucleotide is one which does not normally occur in that animal except in pathological conditions, (i.e. an heterologous protein) such as proteins associated with human immunodeficiency virus, (HIV), the etiologic agent of acquired immune deficiency syndrome, (AIDS), the animals' immune system is activated to launch a protective immune response. Because these exogenous

proteins are produced by the animals' own tissues, the expressed proteins are processed by the major histocompatibility system, MHC, in a fashion analogous to when an actual infection with the related organism, HIV, occurs. The result, as shown in this disclosure, is
5 induction of immune responses against the cognate pathogen.

Accordingly, the instant inventors have prepared nucleic acids which, when introduced into the biological system induce the expression of HIV proteins and epitopes. The induced antibody
10 response is both specific for the expressed HIV protein, and neutralizes HIV. In addition, cytotoxic T-lymphocytes which specifically recognize and destroy HIV infected cells are induced. The instant inventors have also developed polynucleotides whereby simian immunodeficiency virus (SIV) genes are efficiently expressed upon introduction in vivo. This
15 achievement is significant because the only animal model closely mimicking the human disease, AIDS, is the subhuman primate model utilizing SIV. Thus, efficacy of the instant immunogens as vaccines can be shown by analogy to the effects obtained in vivo utilizing HIV and SIV polynucleotide immunogens.

There are many embodiments of the instant invention
20 which those skilled in the art can appreciate from the specifics taught herein. Thus, different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully based on the successful invention disclosed herein.

The instant invention provides a method for using a
25 polynucleotide which, upon introduction into mammalian tissue, induces the co-expression in a single cell, in vivo, of two or more different, discrete gene products. The method is exemplified by using an HIV model which demonstrates the co-expression of more than one gene product in a single cell upon introduction of the polynucleotide into
30 mammalian tissue in vivo. The model is stringent because certain HIV genes contain a sequence known as the REV responsive element (RRE). These genes are not efficiently expressed unless another HIV gene, known as REV, is also present within the cell expressing the RRE-

containing HIV gene. This phenomenon is described as REV dependence.

Pavlakakis and Felber, WO 93/20212 have described a method of eliminating sequences which may induce transcript
5 instability, which may also achieve some REV independence of certain HIV genes. That method may not be generally applicable to all such genes, is time-consuming and may require multiple gene modifications. Furthermore, the level of expression and immunogenicity of such genes may be compromised by elimination of the REV dependence.

10 The instant invention provides a different solution which does not require multiple manipulations of REV dependent HIV genes to obtain REV-independence. In addition, the instant invention is applicable to expression of REV independent genes as well as to expression of REV dependent genes. The REV-dependent expression
15 system described herein, is useful in its own right and is also useful as a stringent system for demonstrating the co-expression in a single cell in vivo of more than a single desired gene-product. Thus, in any circumstance in which it is beneficial to achieve the co-expression, within a given cell in vivo, of more than a single gene product, the
20 methods and polynucleotide constructs described herein may be employed.

One situation, exemplified herein, is the co-expression of an immunogenic epitope and a member of the family of T-cell
25 recognition elements known as B7. Recently, Steven Edgington [Biotechnology 11:1117-1119, 1993] reviewed the coordinate roles of B7 and the major histocompatibility complex (MHC) presentation of epitopes on the surface of antigen presenting cells in activating CD8⁺ CTLs for the elimination of tumors. Once a MHC molecule on the surface of an antigen presenting cell (APC) presents an epitope to a T-
30 cell receptor (TCR), B7 expressed on the surface of the same APC acts as a second signal by binding to CTLA-4 or CD28. The result is rapid division of CD4⁺ helper T-cells which signal CD8⁺ T-cells to proliferate and kill the APC. Thus, our demonstration herein of efficient expression and production of immune responses against an HIV

REV dependent gene containing an RRE by coordinately expressing a gene for REV, conclusively proves that more than one gene can be coordinately expressed by introducing a polynucleotide encoding two and even three cistrons (defined as a stretch of nucleic acid that carries the information for a polypeptide chain).

Because many of the applications of the instant invention apply to anti-viral vaccination, the polynucleotides are frequently referred to as a polynucleotide vaccine (PNV). This is not to say that additional utilities of these polynucleotides, in immune stimulation and in anti-tumor therapeutics, is to be ignored or considered to be outside the scope of the invention.

In one embodiment of this invention, a gene encoding an HIV gene product is incorporated in an expression vector. The vector contains a transcriptional promoter recognized by an eukaryotic RNA polymerase, and a transcriptional terminator at the end of the HIV gene coding sequence. In a preferred embodiment, the promoter is the cytomegalovirus promoter with the intron A sequence (CMV-intA), although those skilled in the art will recognize that any of a number of other known promoters such as the strong immunoglobulin, or other eukaryotic gene promoters may be used. A preferred transcriptional terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator (Fig. 8, SEQ. ID:13:) is particularly preferred. In addition, to assist in preparation of the polynucleotides in prokaryotic cells, an antibiotic resistance marker is also preferably included in the expression vector under transcriptional control of a prokaryotic promoter so that expression of the antibiotic does not occur in eukaryotic cells. Ampicillin resistance genes, neomycin resistance genes or any other pharmaceutically acceptable antibiotic resistance marker may be used. In a preferred embodiment of this invention, the antibiotic resistance gene encodes a gene product for neomycin resistance. Further, to aid in the high level production of the polynucleotide by fermentation in prokaryotic organisms, it is advantageous for the vector to contain a prokaryotic origin of replication and be of high copy number. Any of a number of

commercially available prokaryotic cloning vectors provide these benefits. In a preferred embodiment of this invention, these functionalities are provided by the commercially available vectors known as pUC. It is desirable, however, to remove non-essential DNA sequences. Thus, the lacZ and lacI coding sequences of pUC are removed in one embodiment of the invention. It is also desirable that the vectors not be able to replicate in eukaryotic cells. This minimizes the risk of integration of polynucleotide vaccine sequences into the recipients' genome.

In another embodiment, the expression vector pnRSV is used, wherein the Rous Sarcoma virus (RSV) long terminal repeat (LTR) is used as the promoter. In yet another embodiment, V1, a mutated pBR322 vector into which the CMV promoter and the BGH transcriptional terminator were cloned is used. In a particularly preferred embodiment of this invention, the elements of V1 and pUC19 have been combined to produce an expression vector named V1J (SEQ. ID:12:). Into V1J or another desirable expression vector is cloned an HIV gene, such as gp120, gp41, gp160, gag, pol, env, or any other HIV gene which can induce anti-HIV immune responses (antibody and/or CTLs). Exclusion of functional reverse transcriptase and integrase functions encoded by the HIV genome is desirable to minimize the risk of integration of the polynucleotide vaccine encoded sequences into the recipients' genome. In another embodiment, the ampicillin resistance gene is removed from V1J and replaced with a neomycin resistance gene, to generate V1J-neo (SEQ.ID:14:), into which any of a number of different HIV genes have been cloned for use according to this invention. In yet another embodiment, the vector is V1Jns, which is the same as V1Jneo except that a unique SfiI restriction site has been engineered into the single KpnI site at position 2114 of V1J-neo. The incidence of SfiI sites in human genomic DNA is very low (approximately 1 site per 100,000 bases). Thus, this vector allows careful monitoring for expression vector integration into host DNA, simply by SfiI digestion of extracted genomic DNA. In a further refinement, the vector is V1R. In this vector, as much non-essential

DNA as possible was "trimmed" from the vector to produce a highly compact vector. This vector is a derivative of V1Jns and is shown in Figure 11, (SEQ.ID.:100:). This vector allows larger inserts to be used, with less concern that undesirable sequences are encoded and optimizes uptake by cells when the construct encoding specific influenza virus genes is introduced into surrounding tissue. In figure 11, the portions of V1Jneo (Figure 7) that are deleted are shown as a gap, and inserted sequence is in bold text, but the numbering of V1Jneo is unchanged. The foregoing vector modification and development procedures may be accomplished according to methods known by those skilled in the art. The particular products described however, though obtained by conventional means, are especially useful for the particular purpose to which they are adapted.

One embodiment of this invention incorporates genes encoding HIV gp160, gp120, gag and other gene products from such well known laboratory adapted strains of HIV as SF2, IIIB or MN, for which a great deal of data has been generated, for example, such as showing that chimpanzees can be protected from a lethal challenge of HIV IIIB virus by first administering HIV IIIB V3 loop specific monoclonal antibody [Emini et al., Nature 355: 728-730 1992], or by vaccination with recombinant gp120 but not gp160 [Berman et al., Nature 345 : 822-825, 1990]. Those skilled in the art will recognize that the use of genes from HIV-2 strains having analogous function to the genes from HIV-1 would be expected to generate immune responses analogous to those described herein for HIV-1 constructs. The cloning and manipulation methods for obtaining these genes are well known to those skilled in the art.

There has recently been recognition that elicitation of immune responses against laboratory adapted strains of HIV may not be adequate to provide neutralization of primary, field isolates of HIV, [see for example Cohen, J., Science 262: 980-981, 1993]. Thus, in another embodiment of this invention, genes from virulent, primary field isolates of HIV are incorporated in the polynucleotide immunogen. This is accomplished by preparing cDNA copies of the viral genes and

then subcloning the individual genes into the polynucleotide immunogen. Sequences for many genes of many HIV strains are now publicly available on GENBANK and such primary, filed isolates of HIV are available from the National Institute of Allergy and Infectious Diseases (NIAID) which has contracted with Quality Biological, Inc., [7581 Lindbergh Drive, Gaithersburg, Maryland 20879] to make these strains available. Such strains are also now available from the World Health Organization (WHO) [Network for HIV Isolation and Characterization, Vaccine Development Unit, Office of Research, Global Program on AIDS, CH-1211 Geneva 27, Switzerland]. From this work those skilled in the art will recognize that one of the utilities of the instant invention is to provide a system for in vivo as well as in vitro testing and analysis so that a correlation of HIV sequence diversity with serology of HIV neutralization, as well as other parameters can be made. The isolation and cloning of these various genes may be accomplished according to methods known to those skilled in the art. Thus this invention further provides a method for systematic identification of HIV strains and sequences for vaccine production. Incorporation of genes from primary isolates of HIV strains provides an immunogen which induces immune responses against clinical isolates of the virus and thus meets a need as yet unmet in the field. Furthermore, as the virulent isolates change, the immunogen may be modified to reflect new sequences as necessary.

To keep the terminology consistent, the following convention is followed herein for describing polynucleotide immunogen constructs:

"Vector name-HIV strain-gene-additional elements". Thus, a construct wherein the gp160 gene of the MN strain is cloned into the expression vector V1Jneo, the name it is given herein is: "V1Jneo-MN-gp160". The additional elements that are added to the construct are described in further detail below. Naturally, as the etiologic strain of the virus changes, the precise gene which is optimal for incorporation in the pharmaceutical may be changed. However, as is demonstrated below, because cytotoxic lymphocyte responses are induced which are

capable of protecting against heterologous strains, the strain variability is less critical in the immunogen and vaccines of this invention, as compared with the whole virus or subunit polypeptide based vaccines. In addition, because the pharmaceutical is easily manipulated to insert a new gene, this is an adjustment which is easily made by the standard techniques of molecular biology.

To provide a complete description of the instant invention, the following background on HIV is provided. The human immunodeficiency virus has a ribonucleic acid (RNA) genome, the structure of which is represented in Figure 1. This RNA genome must be reverse transcribed according to methods known in the art in order to produce a cDNA copy for cloning and manipulation according to the methods taught herein. At each end of the genome is a long terminal repeat which acts as a promoter. Between these termini, the genome encodes, in various reading frames, **gag-pol-env** as the major gene products: gag is the group specific antigen; pol is the reverse transcriptase, or polymerase; also encoded by this region, in an alternate reading frame, is the viral protease which is responsible for post-translational processing, for example, of gp160 into gp120 and gp41; env is the envelope protein; vif is the virion infectivity factor; REV is the regulator of virion protein expression; neg is the negative regulatory factor; vpu is the virion productivity factor "u"; tat is the trans-activator of transcription; vpr is the viral protein r. The function of each of these elements has been described (see AIDS 89, A Practical Synopsis of the V International Conference, June 4-9, 1989, Montreal, A Philadelphia Sciences Group Publication, from which figure 1 was adapted).

In one embodiment of this invention, a gene encoding an HIV or SIV protein is directly linked to a transcriptional promoter. The env gene encodes a large, membrane bound protein, gp160, which is post-translationally modified to gp41 and gp120. The gp120 gene may be placed under the control of the cytomegalovirus promoter for expression. However, gp120 is not membrane bound and therefore, upon expression, it may be secreted from the cell. As HIV tends to

remain dormant in infected cells, it is desirable that immune responses directed at cell-bound HIV epitopes also be generated. This goal is accomplished herein by expression in vivo of the cell-membrane associated epitope, gp160, to prime the immune system. However,
5 expression of gp160 is repressed in the absence of REV due to non-export from the nucleus of non-spliced genes. For an understanding of this system, the life cycle of HIV must be described in further detail.

In the life cycle of HIV, upon infection of a host cell, HIV RNA genome is reverse-transcribed into a proviral DNA which
10 integrates into host genomic DNA as a single transcriptional unit. The LTR provides the promoter which transcribes HIV genes from the 5' to 3' direction (gag, pol, env), to form an unspliced transcript of the entire genome. The unspliced transcript functions as the mRNA from which gag and pol are translated, while limited splicing must occur for
15 translation of env encoded genes. For the regulatory gene product REV to be expressed, more than one splicing event must occur because in the genomic setting, REV and env, as is shown in figure 1, overlap. In order for transcription of env to occur, REV transcription must stop, and vice versa. In addition, the presence of REV is required for export
20 of unspliced RNA from the nucleus. For REV to function in this manner, however, a REV responsive element (RRE) must be present on the transcript [Malim et al., Nature 338:254-257 (1989)].

In the polynucleotide vaccine of this invention, the
25 obligatory splicing of certain HIV genes is eliminated by providing fully spliced genes (i.e.: the provision of a complete open reading frame for the desired gene product without the need for switches in the reading frame or elimination of noncoding regions; those of ordinary skill in the art would recognize that when splicing a particular gene, there is
30 some latitude in the precise sequence that results; however so long as a functional coding sequence is obtained, this is acceptable). Thus, in one embodiment, the entire coding sequence for gp160 is spliced, and the sequence of REV is spliced, such that no intermittent expression of each gene product is required. Furthermore, the features of REV regulated

expression are exploited to optimize expression of HIV encoded REV-dependent, immunogenic gene products.

5 For REV to function as an exporter of transcripts from the nucleus to be translated in the cytoplasm, REV requires, in addition to the presence of a REV responsive element (RRE) on the transcript to be exported, at least one splice donor site on the 5' side of the gene containing the RRE [Lu et al., P.N.A.S. USA 87:7598-7602, (October 1990); Chang and Sharp, Cell 59:789-795 (December 1, 1989)]. The instant inventors conceived polynucleotides providing the REV coding sequence in a location on the same expression vector as the gene to be expressed such that co-expression of REV and the REV responsive gene occur without the need for any splicing. Thus, in a preferred embodiment of this invention, HIV genes are placed immediately downstream from a transcriptional promoter, such as the CMV promoter, and the spliced REV coding sequence is placed at a location 3' to (also referred to as downstream from) the first coding sequence. Naturally, the order of these genes could be changed. However, it may be preferable to have the immunogenic HIV cistron abut directly to the transcriptional promoter to ensure that all transcripts produced encode the entire cistron.

One method for achieving co-expression of genes relies on co-transfection of cells in culture with different vectors expressing different genes. For a REV dependent gene, the REV gene product could be provided in this manner in trans. However, this is suboptimal for the purposes of this invention, although not outside the scope of the instant invention, because of the low probability that co-transfection of a given cell would occur in vivo so as to achieve the necessary availability of REV for vigorous expression of REV dependent immunogenic HIV gene products. Another method is to provide several promoters on a given vector, each promoter controlling expression of a separate gene. This amounts to providing REV gene product in cis. This solution may be employed according to the instant invention. In such an embodiment, it would be preferable for the various promoters and the genes they control to run in opposite directions. However, because of the known

competitive interference between promoters in this type of multiple gene vector, this embodiment is also considered sub-optimal.

5 Ghattas et al., [Mol. and Cell. Biol. 11, No. 12:5848-5859 (Dec. 1991)], Kaufaman et al. [Nuc. Acids Res. 19, No. 16:4485-4490 (1991)], and Davies [J. Virol. 66, No. 4:1924-1932 (Apr. 1992)] have described an internal ribosome entry site (IRES) in the encephalomyocarditis virus (EMCV) leader. They reported that a system in which an upstream promoter could be used to initiate
10 transcription of a dicistronic mRNA provides good expression of both the 5' and 3' open reading frames when an IRES is located between the two genes. Chen et al. (J. Viral., 67 : 2142-2145, 1993] have reported a system in which the 5 nontranslated region (NTR) from swine vesicular disease virus (SVDV) was used to construct a bicistronic virus for the
15 coexpression of two genes from one transcript from an infectious viral vector.

The instant inventors have discovered that a nucleic acid construct which incorporates coordinated expression of an HIV gene containing a REV responsive element (RRE), an internal ribosome entry site (IRES) and a REV coding sequence results in efficient expression of
20 both REV and the REV dependent gene product. This embodiment of the invention is better understood with reference to figures 2 and 3. Fig. 2 shows a generalized embodiment while, Figure 3, shows a specific embodiment of this invention which, according to the nomenclature system described above, is V1Jns-gp160(RRE)-IRES-
25 REV. The strain of HIV from which the immunogenic HIV gene is derived is irrelevant for the illustrative purposes of this discussion, and indeed, the expression of any REV dependent gene product is predictably efficient, as is the elicitation of immune responses against both REV and the REV dependent gene product, based on the instant
30 patent disclosure. According to the embodiment shown in Fig. 3, the vector is V1Jns, described above. Thus, the promoter (CMVintA) and terminator (BGH) are provided for by the vector, along with a prokaryotic origin of replication, to facilitate large scale production of the HIV polynucleotide vaccine through fermentation of bacteria

transformed with the construct, according to methods well known in the art. This construct does not replicate in eukaryotic tissue, due to the absence of an eukaryotic origin of replication. A splice donor site from the naturally occurring rev/tat splice donor is provided (rev/tat SD)
5 immediately preceding the HIV gene. The gag/pol/env coding sequence contains or is followed by a REV responsive element (RRE) which, upon formation of the nascent transcript, provides the necessary signals for REV binding to and export of the REV dependent mRNA from the nucleus. Next, there are sequences provided for reinitiation of
10 translation at the internal ribosome entry site (IRES) so that the downstream REV coding sequence is efficiently translated. In this manner, REV gene product is provided in cis, on the same polynucleotide as a REV dependent gene product.

In further refinements to the instant invention, a third
15 cistron may be included in the PNV. The genes encoding such immunostimulatory proteins as the B7-antigen presenting cell-surface protein, the human granulocyte/monocyte colony stimulatory factor (GM-CSF) gene, and cytokine genes such as interleukin and interferon, the use of tissue-specific transcriptional promoters and enhancers, are
20 all contemplated. The provision of B7 or GM-CSF gene in cis, either by insertion of an IRES after REV and before the B7 gene, by provision of a second promoter on the same vector construct as the dicistronic REV-dependent HIV gene, IRES-REV construct, or in trans using a separate construct are all envisioned by extension of the foregoing
25 teachings regarding REV and REV dependent genes. The generalized immuno-stimulatory effect of these gene products may be sufficient even if provided in trans to enhance immune responses against the HIV gene products encoded by the immunogen of this invention. It is
30 preferable, particularly for B7, that the same cell presenting HIV epitopes in the cleft of MHC-I molecules also present B7. This co-presentation of both the antigenic epitope and B7 "closes" the switch necessary for T-cell activation. Cytokines, particularly IL-12, which modifies whether a predominant humoral or cellular immune response is mounted [see Afonso et al., Science 263:235-237, 1994], either is

provided intravenously at the same time that PNV is introduced, or is included as a third cistron in the PNV, thereby assuring localized production of the interleukin. The genes for these immunostimulatory and immunoregulatory proteins, including GM-CSF (see Shaw and Kamen, Cell 46:659-667, 1986), interleukin-12 (see Wolf, S., et al., J. Immunol. 146:3074-3081, 1991) and B7, (see Gordon et al., J. Immunol. 143:2714-2722, 1989; for clones and sequences of newer members of the B7 family of proteins, see also Azuma, M., et al., Nature 366:76-79, 1993; and Freeman, G., et al., Science 262:909-911, 1993) are known and easily cloned and incorporated in PNV's according to this invention using methods known to the skilled practitioner. Preferably, the genes used for these purposes are the human genes so that immune responses against these proteins are minimized, allowing the expressed proteins to carry out their immunomodulatory and immunostimulatory functions. Where HIV genes have been rendered REV-independent, the REV cistron may be eliminated completely and a second cistron encoding a B7 gene family member and a third cistron encoding yet another gene-product such as IL-12, may be constructed.

The use of tissue-specific promoters or enhancers, for example the muscle creatine kinase (MCK) enhancer element, is desirable whenever it is desirable to limit expression of the polynucleotide to a particular tissue type. For example, myocytes are terminally differentiated cells which do not divide. Integration of foreign DNA into chromosomes appears to require both cell division and protein synthesis. Thus, limiting protein expression to non-dividing cells such as myocytes is preferable. However, use of the CMV promoter is adequate for achieving expression in many tissues into which the PNV is introduced.

In the various embodiments of this invention which are described below, the basic paradigm described above is used. Deviations, additions or subtractions from this basic construction design serve to highlight the various aspects of this invention.

This patent disclosure exemplifies bi- or tri-cistronic HIV polynucleotide immunogens as polynucleotide vaccines, PNVs, to generate humoral immunity as well as cross-strain cellular antiviral immunity. The system is useful, however, for any two or three cistrons, whether or not related to HIV, when co-expression of the encoded gene products in a single cell in vivo is required. However, the dual humoral and cellular immune responses generated according to this invention are particularly significant to inhibiting HIV infection, given the propensity of HIV to mutate within the infected population, as well as in infected individuals. In order to formulate an effective protective vaccine for HIV it is desirable to generate both a multivalent antibody response for example to gp160 (*env* is approximately 80% conserved across various HIV-1, clade B strains, which are the prevalent strains in US human populations), the principal neutralization target on HIV, as well as cytotoxic T cells reactive to the conserved portions of gp160 and, internal viral proteins encoded by *gag*. We have made an HIV vaccine comprising gp160 genes selected from common laboratory strains; from predominant, primary viral isolates found within the infected population; from mutated gp160s designed to unmask cross-strain, neutralizing antibody epitopes; from other representative HIV genes such as the *gag* gene ($\geq 95\%$ conserved across HIV isolates); and from SIV, which provides an animal model for testing the HIV PNV wherein non-human primates can be immunized and challenged to test viral load and progression to disease.

Virtually all HIV seropositive patients who have not advanced towards an immunodeficient state harbor anti-*gag* CTLs while about 60% of these patients show cross-strain, gp160-specific CTLs. The amount of HIV specific CTLs found in infected individuals that have progressed on to the disease state known as AIDS, however, is much lower, demonstrating the significance of our findings that we can induce cross-strain CTL responses. Because HIV late gene expression is *REV* dependent our gp160 and *gag* vaccination vectors are designed to also produce *REV* ($\sim 90\%$ conserved), to facilitate the *REV*-dependent gene expression. An additional benefit of this invention is that anti-

REV immune responses are also generated. This gives further advantage to our vaccine because *REV* is made in large quantities very early following infection of a cell, and hours in advance of synthesis of the late gene products, thereby providing an earlier opportunity for intervention by vaccine-induced T-cell responses including CTLs and T-helper cells.

In a further embodiment of this invention, a cocktail vaccine is prepared in which different HIV REV-dependent gene constructs are mixed together to generate anti-REV CTL responses in addition to antibodies and CTL against the immunogenic HIV REV-dependent gene products. According to this embodiment, one polynucleotide encoding gp160, followed by REV, followed by B7, in a tri-cistronic construct having one promoter and two IRES sequences, is mixed with another polynucleotide encoding a *gag* gene product, REV, and B7 or another immunomodulatory or immunostimulatory gene product such as IL-12 or GM-CSF. In this fashion, with a single or several injections of polynucleotide, immune responses against several HIV related immunogens can be raised. Likewise, one polynucleotide comprising a REV independent gene product, such as those described in WO 93/20212, B7, and another immunomodulatory or immunostimulatory gene, such as IL-12 or GM-CSF, are mixed with another REV-dependent, or REV-independent bi- or tri-cistronic expression construct. Furthermore, multiple bi- or tri-cistronic constructs encoding HIV or other antigens could be prepared and mixed to produce a multivalent combination polynucleotide vaccine.

Immune responses induced by our *env*, *REV*, and *gag* polynucleotide vaccine constructs are demonstrated in mice, rabbits, and primates. Monitoring antibody production to *env* in mice allows confirmation that a given construct is suitably immunogenic, i.e., a high proportion of vaccinated animals show an antibody response. Mice also provide the most facile animal model suitable for testing CTL induction by our constructs and are therefore used to evaluate whether a particular construct is able to generate such activity. However, mouse cell lines have been observed to not support efficient *REV* or *tat*

functions. This observation was made in the context of HIV LTR driven expression of late genes and a limited amount of data indicates that heterologous promoters allow *REV* function in mouse cells. Rabbits and monkeys (African Green, rhesus, chimpanzees) provide additional species including primates for antibody evaluation in larger, non-rodent animals. These species are also preferred to mice for antisera neutralization assays due to high levels of endogenous neutralizing activities against retroviruses observed in mouse sera. These data demonstrate that sufficient immunogenicity is engendered by our vaccines to achieve protection in experiments in a chimpanzee/HIVIII_B challenge model. The currently emerging and increasingly accepted definition of protection in the scientific community is moving away from so-called "sterilizing immunity", which indicates complete protection from HIV infection, to prevention of disease. A number of correlates of this goal include reduced blood viral titer, as measured either by HIV reverse transcriptase activity, by infectivity of samples of serum, by ELISA assay of p24 or other HIV antigen concentration in blood, increased CD4⁺ T-cell concentration, and by extended survival rates [see, for example, Cohen, J., Science 262:1820-1821, 1993, for a discussion of the evolving definition of anti-HIV vaccine efficacy]. The immunogens of the instant invention also generate neutralizing immune responses against infectious (clinical, primary field) isolates of HIV.

Immunology

A. Antibody Responses to *env*.

1. gp160 and gp120. An ELISA assay is used to determine whether vaccine vectors expressing either secreted gp120 or membrane-bound gp160 are efficacious for production of *env*-specific antibodies. Initial in vitro characterization of *env* expression by our vaccination vectors is provided by immunoblot analysis of gp160 transfected cell lysates. These data confirm and quantitate gp160 expression using anti-gp41 and anti-gp120 monoclonal antibodies to visualize transfectant cell gp160 expression. In one embodiment of this invention, gp160 is

preferred to gp120 for the following reasons: (1) an initial gp120 vector gave inconsistent immunogenicity in mice and was very poorly or non-responsive in African Green Monkeys; (2) gp160 contributes additional neutralizing antibody as well as CTL epitopes by providing the addition of approximately 190 amino acid residues due to the inclusion of gp41; (3) gp160 expression is more similar to viral *env* with respect to tetramer assembly and overall conformation; and (4) we find that, like the success of membrane-bound, influenza HA constructs for producing neutralizing antibody responses in mice, ferrets, and nonhuman primates [see Ulmer et al., *Science* 259:1745-1749, 1993; Montgomery, D., et al., *DNA and Cell Biol.* 12:777-783, 1993] anti-gp160 antibody generation is superior to anti-gp120 antibody generation. Selection of which type of *env*, or whether a cocktail of *env* subfragments, is preferred is determined by the experiments outlined below.

2. Presence and Breadth of Neutralizing Activity. ELISA positive antisera from rabbits and monkeys is tested and shown to neutralize both homologous and heterologous HIV strains.

3. V3 vs. non-V3 Neutralizing Antibodies. A major goal for *env* PNVs is to generate broadly neutralizing antibodies. It has now been shown that antibodies directed against V3 loops are very strain specific, and the serology of this response has been used to define strains.

a. Non-V3 neutralizing antibodies appear to primarily recognize discontinuous, structural epitopes within gp120 which are responsible for CD4 binding. Antibodies to this domain are polyclonal and more broadly cross-neutralizing probably due to restraints on mutations imposed by the need for the virus to bind its cellular ligand. An in vitro assay is used to test for blocking gp120 binding to CD4 immobilized on 96 well plates by sera from immunized animals. A second in vitro assay detects direct antibody binding to synthetic peptides representing selected V3 domains immobilized on plastic.

These assays are compatible for antisera from any of the animal types used in our studies and define the types of neutralizing antibodies our vaccines have generated as well as provide an in vitro correlate to virus neutralization.

5 b. gp41 harbors at least one major neutralization determinant, corresponding to the highly conserved linear epitope recognized by the broadly neutralizing 2F5 monoclonal antibody (commercially available from Viral Testing Systems Corp., Texas
10 Commerce Tower, 600 Travis Street, Suite 4750, Houston, TX 77002-3005(USA), or Waldheim Pharmazeutika GmbH, Boltzmannsgasse 11, A-1091 Wien, Austria), as well as other potential sites including the well-conserved "fusion peptide" domain located at the N-terminus of gp41. Besides the detection of antibodies directed against gp41 by immunoblot
15 as described above, an in vitro assay test is used for antibodies which bind to synthetic peptides representing these domains immobilized on plastic.

4. Maturation of the Antibody Response. In HIV
20 seropositive patients, the neutralizing antibody responses progress from chiefly anti-V3 to include more broadly neutralizing antibodies comprising the structural gp120 domain epitopes described above (#3), including gp41 epitopes. These types of antibody responses are monitored over the course of both time and subsequent vaccinations.

25 B. T Cell Reactivities Against *env*, *REV*, *nef* and *gag*.

1. Generation of CTL Responses. Viral proteins which are
synthesized within cells give rise to MHC I-restricted CTL responses. Each of these proteins elicit CTL in seropositive patients. Our vaccines
30 also are able to elicit CTL in mice. The immunogenetics of mouse strains are conducive to such studies, as demonstrated with influenza NP, [see Ulmer et al., Science 259:1745-1749, 1993]. Several epitopes have been defined for the HIV proteins *env*, *REV*, *nef* and *gag* in Balb/c mice, thus facilitating in vitro CTL culture and cytotoxicity assays. Additionally, it is advantageous to use syngenic tumor lines,

such as the murine mastocytoma P815, transfected with these genes to provide targets for CTL as well as for in vitro antigen specific restimulation. Methods for defining immunogens capable of eliciting MHC class I-restricted cytotoxic T lymphocytes are known [see Calin-Laurens, et al., Vaccine 11(9):974-978, 1993; see particularly Eriksson, et al., Vaccine 11(8):859-865, 1993, wherein T-cell activating epitopes on the HIV gp120 were mapped in primates and several regions, including gp120 amino acids 142-192, 296-343, 367-400, and 410-453 were each found to induce lymphoproliferation; furthermore, discrete regions 248-269 and 270-295 were lymphoproliferative. A peptide encompassing amino acids 152-176 was also found to induce HIV neutralizing antibodies], and these methods may be used to identify immunogenic epitopes for inclusion in the PNV of this invention. Alternatively, the entire gene encoding gp160, gp120, protease, or gag could be used. For additional review on this subject, see for example, Shirai et al., J. Immunol 148:1657-1667, 1992; Choppin et al., J. Immunol 147:569-574, 1991; Choppin et al., J. Immunol 147:575-583, 1991; Berzofsky et al., J. Clin. Invest. 88:876-884, 1991. As used herein, T-cell effector function is associated with mature T-cell phenotype, for example, cytotoxicity, cytokine secretion for B-cell activation, and/or recruitment or stimulation of macrophages and neutrophils.

2. Measurement of TH Activities. Spleen cell cultures derived from vaccinated animals are tested for recall to specific antigens by addition of either recombinant protein or peptide epitopes. Activation of T cells by such antigens, presented by accompanying splenic antigen presenting cells, APCs, is monitored by proliferation of these cultures or by cytokine production. The pattern of cytokine production also allows classification of TH response as type 1 or type 2. Because dominant TH2 responses appear to correlate with the exclusion of cellular immunity in immunocompromised seropositive patients, it is possible to define the type of response engendered by a given PNV in patients, permitting manipulation of the resulting immune responses.

3. Delayed Type Hypersensitivity (DTH). DTH to viral antigen after i.d. injection is indicative of cellular, primarily MHC II-restricted, immunity. Because of the commercial availability of recombinant HIV proteins and synthetic peptides for known epitopes, DTH responses are easily determined in vaccinated vertebrates using these reagents, thus providing an additional in vivo correlate for inducing cellular immunity.

Protection

Based upon the above immunologic studies, it is predictable that our vaccines are effective in vertebrates against challenge by virulent HIV. These studies are accomplished in an HIV_{IIIB}/chimpanzee challenge model after sufficient vaccination of these animals with a PNV construct, or a cocktail of PNV constructs comprised of gp160_{IIIB}, gag_{IIIB}, nef_{IIIB} and REV_{IIIB}. The _{IIIB} strain is useful in this regard as the chimpanzee titer of lethal doses of this strain has been established. However, the same studies are envisioned using any strain of HIV and the epitopes specific to or heterologous to the given strain. A second vaccination/challenge model, in addition to chimpanzees, is the *scid*-hu PBL mouse. This model allows testing of the human lymphocyte immune system and our vaccine with subsequent HIV challenge in a mouse host. This system is advantageous as it is easily adapted to use with any HIV strain and it provides evidence of protection against multiple strains of primary field isolates of HIV. A third challenge model utilizes hybrid HIV/SIV viruses (SHIV), some of which have been shown to infect rhesus monkeys and lead to immunodeficiency disease resulting in death [see Li, J., et al., J. AIDS 5:639-646, 1992]. Vaccination of rhesus with our polynucleotide vaccine constructs is protective against subsequent challenge with lethal doses of SHIV.

PNV Construct Summary

HIV and other genes are preferably ligated into an expression vector which has been specifically optimized for polynucleotide vaccinations. According to this invention disclosure, methods for producing several such vectors are enabled. Essentially, all extraneous DNA is removed, leaving the essential elements of transcriptional promoter, immunogenic epitopes, and additional cistrons encoding immunoenhancing or immunomodulatory genes, with their own promoters or IRES, transcriptional terminator, bacterial origin of replication and antibiotic resistance gene, as previously described (see figure 2). Those skilled in the art will appreciate that introduction of RNA which has been transcribed in vitro to produce the multi-cistronic mRNAs encoded by the DNA counterparts of this invention naturally forms an integral part of this invention. For this purpose, it is desirable to use as the transcriptional promoter such powerful RNA polymerase promoters as the T7 or SP6 promoters, and performing run-on transcription with a linearized DNA template. These methods are well known in the art.

Expression of HIV late genes such as *env* and *gag* is *REV*-dependent and requires that the *REV* response element (RRE) be present on the viral gene transcript. A secreted form of gp120 can be generated in the absence of *REV* by substitution of the gp120 leader peptide with a heterologous leader such as from tPA (tissue-type plasminogen activator), and preferably by a leader peptide such as is found in highly expressed mammalian proteins such as immunoglobulin leader peptides. We have inserted a tPA-gp120 chimeric gene into V1Jns which efficiently expresses secreted gp120 in transfected cells (RD, a human rhabdomyosarcoma line). We have also developed an IRES-based (IRES = internal ribosomal entry site) dicistronic V1Jns vector containing both gp160 (which harbors the RRE) and *REV* which efficiently expresses gp160 in transfected cell lines (293, a human embryonic kidney cell line; and RD). Monocistronic gp160 does not produce any protein upon transfection without the addition of a *REV* expression vector. Dicistronic gp160/*REV* produces similar amounts of gp160 as co-transfected gp160 and *REV* monocistronic vectors.

From these studies, it is predictable that dicistronic vectors more efficiently express gp160 following introduction in vivo intramuscularly relative to a mixture of gp160 and *REV* vectors because the dicistron insures the proximity of gp160 construct and *REV* within structurally extended, multi-nucleated muscle cells. This dicistronic strategy also supports expression of *gag* after the inclusion of the RRE within the transcript region of the vector. It also supports the expression of unrelated genes in a bi- or tri-cistronic PNV, such as co-expression of HIV immunogenic epitopes, influenza virus immunogenic epitopes, cancer-related antigens, and immunomodulatory genes such as interleukin, B7 and GM-CSF.

Representative Construct Components Include (but are not restricted to) (see figure 2, Cistrons I, II, and III):

1. tPA-gp120MN;
2. gp160IIIB/TRES/*REV*IIIB;
3. gp160IIIB;
4. *REV*IIIB;
5. *tat/REV/gp160* (a genomic IIIB clone which weakly expresses gp160);
6. *REV/gp160*;
7. gp160MN;
8. gp160 from clinically relevant primary HIV isolates;
9. *nef*, using the gene from clinically relevant strains;
10. *gag*IIIB: for anti-*gag* CTL;
11. tPA-gp120IIIB: for chimp studies;
12. gp160 with structural mutations: V3 loop substitutions from clinically relevant strains of HIV; several mutations on several constructs such as variable loop removal, Asn mutations to remove steric carbohydrate obstacles to structural, neutralizing antibody epitopes; and CD4 binding site knockout mutants;
13. gp41: to specifically elicit anti-gp41 neutralizing

antibodies, particularly the 2F5 monoclonal antibody epitope, located directly anterior to the transmembrane domain, which is broadly conserved across many strains. This peptide is difficult to express in the absence of gp120 and requires several strategies, e.g., a recent report found that the 2F5 epitope spliced into an influenza HA loop tip could elicit HIV neutralizing antibodies; alternatively, provision of appropriate leader sequences, as in the tPA signal peptide leader sequence, allows expression of this gene product;

14. *gag*: similar to construct from #5 above, using the gene from clinically relevant strains;

15. *rev*: for gp160 and *gag* dicistronics;

16. B7 coding sequences;

17. GM-CSF sequences;

18. Interleukin sequences, particularly encoding IL-12;

19. Tumor associated antigens;

20. Genes encoding antigens expressed by pathogens other than HIV, such as, but not limited to, influenza virus nucleoprotein, hemagglutinin, matrix, neuraminidase, and other antigenic proteins; herpes simplex virus genes; human papillomavirus genes; tuberculosis antigens; hepatitis A, B, or C virus antigens; and combinations of these and other antigens to form at least dicistronic constructs which may be combined with multiple other polycistronic constructs to provide a cocktail composition capable of raising immune responses against all of the represented pathogens or tumor antigens.

30 In the HIV *env* constructs, those of ordinary skill in the art will recognize the desirability of expressing nucleic acids encoding various *env* V3 loop amino acid sequences. As an example, any or all of the following amino acid sequences, or portions thereof, may be encoded by HIV polynucleotide immunogens of this invention:

GP160 V3 LOOP SEQUENCE SUMMARY FOR PNV CONSTRUCTS

- 5 North American/European Consensus, SEQ.ID:1:
 CysThrArgProAsnAsnAsnThrArgLysSerIleHisIle**GlyProGlyArgAla**
 PheTyrThrThrGlyGluIleIleGlyAspIleArgGlnAlaHisCys
- MN, SEQ.ID:2:
 10 CysThrArgProAsnTyrAsnLysArgLysArgIleHisIle**GlyProGlyArgAla**
 PheTyrThrThrLysAsnIleIleGlyThrIleArgGlnAlaHisCys
- IIIB (HXB2R), SEQ.ID:3:
 15 CysThrArgProAsnAsnAsnThrArgLysArgIleArgIleGlnArg**GlyProGly**
ArgAlaPheValThrIleGlyLysIleGlyAsnMetArgGlnAlaHisCys
- 116-v, SEQ.ID:4:
 CysThrArgProAsnAsnAsnThrArgLysGlyIleHisIle**GlyProGlyArgAla**
 PheTyrThrThrGlyLysIleIleGlyAsnIleArgGlnAlaHisCys
- 20 452-p, SEQ.ID:5:
 CysThrArgProSerAsnAsnAsnThrArgLysSerIleHisIle**GlyProGlyLys**
 AlaPheTyrAlaThrGlyAlaIleIleGlyAspIleArgGlnAlaHisCys
- 25 146-v, SEQ.ID:6:
 CysThrArgProAsnAsnAsnThrArgArgSerIleHisIle**AlaProGlyArgAla**
PheTyrAlaThrGlyAspIleIleGlyAspIleArgGlnAlaHisCys

- 30 The protective efficacy of polynucleotide HIV immunogens
 against subsequent viral challenge is demonstrated by immunization with
 the non-replicating plasmid DNA of this invention. This is
 advantageous since no infectious agent is involved, no assembly of virus
 particles is required, and determinant selection is permitted.
 Furthermore, because the sequence of *gag* and protease and several of
 the other viral gene products is conserved among various strains of

HIV, protection against subsequent challenge by a virulent strain of HIV that is homologous to, as well as strains heterologous to the strain from which the cloned gene is obtained, is enabled.

5 The i.m. injection of a DNA expression vector encoding
gp160 results in the generation of significant protective immunity
against subsequent viral challenge. In particular, gp160-specific
antibodies and primary CTLs are produced. Immune responses directed
against conserved proteins can be effective despite the antigenic shift
10 and drift of the variable envelope proteins. Because each of the HIV
gene products exhibit some degree of conservation, and because CTLs
are generated in response to intracellular expression and MHC
processing, it is predictable that many virus genes give rise to responses
analogous to that achieved for gp160. Thus, many of these genes have
15 been cloned, as shown by the cloned and sequenced junctions in the
expression vector (see below) such that these constructs are
immunogenic agents in available form.

 The invention offers a means to induce cross-strain
protective immunity without the need for self-replicating agents or
adjuvants. In addition, immunization with the instant polynucleotides
20 offers a number of other advantages. First, this approach to vaccination
should be applicable to tumors as well as infectious agents, since the
CD8⁺ CTL response is important for both pathophysiological processes
[K. Tanaka *et al.*, Annu. Rev. Immunol. 6, 359 (1988)]. Therefore,
eliciting an immune response against a protein crucial to the
25 transformation process may be an effective means of cancer protection
or immunotherapy. Second, the generation of high titer antibodies
against expressed proteins after injection of viral protein and human
growth hormone DNA, [see for example D.-c. Tang *et al.*, Nature 356,
152, 1992], indicates this is a facile and highly effective means of
30 making antibody-based vaccines, either separately or in combination
with cytotoxic T-lymphocyte vaccines targeted towards conserved
antigens.

 The ease of producing and purifying DNA constructs
compares favorably with traditional protein purification, facilitating the

generation of combination vaccines. Thus, multiple constructs, for example encoding gp160, gp120, gp41, or any other HIV gene may be prepared, mixed and co-administered. Finally, because protein
5 expression is maintained following DNA injection [H. Lin *et al.*, Circulation **82**, 2217 (1990); R.N. Kitsis *et al.*, Proc. Natl. Acad. Sci. (USA) **88**, 4138 (1991); E. Hansen *et al.*, FEBS Lett. **290**, 73 (1991); S. Jiao *et al.*, Hum. Gene Therapy **3**, 21 (1992); J.A. Wolff *et al.*, Human Mol. Genet. **1**, 363 (1992)], the persistence of B- and T-cell
10 memory may be enhanced [D. Gray and P. Matzinger, J. Exp. Med. **174**, 969 (1991); S. Oehen *et al.*, *ibid.* **176**, 1273 (1992)], thereby engendering long-lived humoral and cell-mediated immunity.

The standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the
15 DNA immunogens of this invention. While standard techniques of molecular biology are therefore sufficient for the production of the products of this invention, the specific constructs disclosed herein provide polynucleotide immunogens which surprisingly produce cross-strain and primary HIV isolate neutralization, a result heretofore
20 unattainable with standard inactivated whole virus or subunit protein vaccines.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the
25 immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 µg to 300 µg is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as
30 intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided. Following vaccination with HIV polynucleotide immunogen, boosting with HIV protein immunogens such as gp160, gp120, and gag gene products is also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of

administration of interleukin-12 protein, concurrently with or subsequent to parenteral introduction of the PNV of this invention also advantageous.

5 The polynucleotide may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipients' immune system. In this case, it is desirable for the polynucleotide to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the DNA may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture, or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers. Techniques for coating microprojectiles coated with polynucleotide are known in the art and are also useful in connection with this invention.

20 Accordingly, one embodiment of this invention is a polynucleotide which, upon introduction into mammalian tissue, induces the co-expression in a single cell, in vivo, of two or three different, discrete gene products, comprising:

25 a first transcriptional promoter which operates efficiently in eukaryotic cells upstream from and in transcriptional control of a first cistron;
a second cistron downstream from the first cistron, under transcriptional control either of the first transcriptional promoter, or under control of a second transcriptional promoter;
optionally, a third cistron downstream from the second cistron, under transcriptional control either of the first transcriptional promoter,
30 under control of a second transcriptional promoter, or under control of a third transcriptional promoter;
a transcriptional terminator following each of the first, second and third cistron, unless followed by another cistron lacking its own transcriptional promoter.

In another embodiment, the invention is a polynucleotide which comprises contiguous nucleic acid sequences which cannot replicate in eukaryotic cells but which are capable of being expressed to produce a gene product upon introduction of the polynucleotide into eukaryotic tissues in vivo. The encoded gene product preferably either acts as an immunostimulant or as an antigen capable of generating an immune response. Thus, the nucleic acid sequences in this embodiment encode a spliced REV gene, a human immunodeficiency virus (HIV) immunogenic epitope, and optionally, a cytokine or a T-cell costimulatory element, such as a member of the B7 family of proteins.

In another embodiment, the invention is a method for co-expression in a single cell, in vivo, of two or three different, discrete gene products, which comprises introducing between about 0.1 µg and 100 mg of a polynucleotide of this invention into the tissue of the vertebrate.

In another embodiment, the invention is a method for using a REV dependent HIV gene to induce immune responses in vivo which comprises:

- a) isolating the REV dependent HIV gene;
- b) linking the isolated gene to regulatory sequences such that the gene is expressible by virtue of being operatively linked to control sequences which, when introduced into a living tissue, direct the transcription initiation and subsequent translation of the gene;
- c) introducing the expressible gene into a living tissue;
- d) introducing a gene encoding HIV REV either in trans or in cis to the HIV REV dependent gene; and
- e) optionally, boosting with additional expressible HIV gene.

A further embodiment of this invention amounts to a method of inducing an antigen presenting cell to stimulate cytotoxic T-cell proliferation specific to HIV antigens. This involves exposing cells of a vertebrate in vivo to a polynucleotide which consists of an antigenic HIV epitope, REV if the antigenic HIV epitope depends on REV for efficient expression, and B7 encoding sequences.

[see B.S. Chapman *et al.*, *ibid.*,] which includes hCMV-IE1 enhancer/promoter and intron A, into the Hind III and Xba I sites of pBL3 to generate pCMVIntBL. The 1881 base pair luciferase gene fragment (Hind III-Sma I Klenow filled-in) from RSV-Lux [J.R. de Wet *et al.*, Mol. Cell Biol. 7, 725, 1987] was cloned into the Sal I site of pCMVIntBL, which was Klenow filled-in and phosphatase treated.

The primers that spanned intron A are:

5' primer, SEQ. ID:7:

5'-CTATATAAGCAGAG CTCGTTTAG-3'; The 3' primer, SEQ ID:8:
5'-GTAGCAAAGATCTAAGGACGGTGA CTGCAG-3'.

The primers used to remove the Sac I site are:

sense primer, SEQ ID:9:

5-GTATGTGTCTGAAAATGAGCGTGGAGATTGGGCTCGCAC-3'

and the antisense primer, SEQ ID:10:,

5'-GTGCGAGCCCAATCTCCACGCTCATTTTCAGACACA TAC-3'.

The PCR fragment was cut with Sac I and Bgl II and inserted into the vector which had been cut with the same enzymes.

B) V1J EXPRESSION VECTOR, SEQ. ID:12:

Our purpose in creating V1J was to remove the promoter and transcription termination elements from our vector, V1, in order to place them within a more defined context, create a more compact vector, and to improve plasmid purification yields.

V1J is derived from vectors V1, (see Example 1) and pUC18, a commercially available plasmid. V1 was digested with SspI and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes (SEQ ID:13:), was purified from an agarose electrophoresis gel. The ends of this DNA

fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment.

5 pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of minimum size. We removed the entire *lac* operon from this vector, which was unnecessary for our purposes and may be detrimental to plasmid yields and heterologous gene expression, by partial digestion with the HaeII
10 restriction enzyme. The remaining plasmid was purified from an agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase, treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC
15 backbone were obtained. One of these plasmids gave much higher yields of DNA in *E. coli* and was designated V1J (SEQ. ID:12:). This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with V1.

20 C) V1Jneo EXPRESSION VECTOR, SEQ. ID:14:

It was necessary to remove the *amp^r* gene used for antibiotic selection of bacteria harboring V1J because ampicillin may not be used in large-scale fermenters. The *amp^r* gene from the pUC
25 backbone of V1J was removed by digestion with SspI and Eam1105I restriction enzymes. The remaining plasmid was purified by agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and then treated with calf intestinal alkaline phosphatase. The commercially available *kan^r* gene, derived from transposon 903 and contained within
30 the pUC4K plasmid, was excised using the PstI restriction enzyme, purified by agarose gel electrophoresis, and blunt-ended with T4 DNA polymerase. This fragment was ligated with the V1J backbone and plasmids with the *kan^r* gene in either orientation were derived which were designated as V1Jneo #'s 1 and 3. Each of these plasmids was confirmed by restriction enzyme digestion analysis, DNA sequencing of

the junction regions, and was shown to produce similar quantities of plasmid as V1J. Expression of heterologous gene products was also comparable to V1J for these V1Jneo vectors. We arbitrarily selected V1Jneo#3, referred to as V1Jneo hereafter (SEQ. ID:14:), which
5 contains the kan^r gene in the same orientation as the amp^r gene in V1J as the expression construct.

D) V1Jns EXPRESSION VECTOR:

10 An Sfi I site was added to V1Jneo to facilitate integration studies. A commercially available 13 base pair Sfi I linker (New England BioLabs) was added at the Kpn I site within the BGH sequence of the vector. V1Jneo was linearized with Kpn I, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt Sfi I linker. Clonal
15 isolates were chosen by restriction mapping and verified by sequencing through the linker. The new vector was designated V1Jns. Expression of heterologous genes in V1Jns (with Sfi I) was comparable to expression of the same genes in V1Jneo (with Kpn I).

20 E) pGEM-3-IRES: The encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) allows efficient expression of two genes within a single mRNA transcript when it is juxtaposed between them. We have utilized this non-coding gene segment to create dicistronic expression vectors for polynucleotide vaccines. The EMCV IRES
25 segment was subcloned as a 0.6 kb EcoR1/BssHII digestion fragment from the pCITE-1 plasmid (Novagen). This fragment was agarose gel-purified, blunt-ended using T4 DNA polymerase and subsequently ligated into pGEM-3 (Promega) which had been XbaI-digested, blunt-ended with T4 DNA polymerase, and phosphatased. Clones were
30 obtained for each of the two possible orientations of this DNA within pGEM-3 and each junction site verified by DNA sequencing. The preferred orientation for subsequent construction of dicistronic vectors positioned the NcoI site within the IRES proximal to BamHI site within pGEM-3. This vector is referred to as pGEM-3-IRES.

F) pGEM-3-IRES*: A second IRES vector was prepared containing mutations in the IRES sequence (IRES*) conferred by a PCR oligomer which may optimize IRES-driven expression compared to wild type IRES. PCR amplification of IRES* was performed using pCITE-1
 5 plasmid (Novagen) with the following sense and antisense oligomers: 5'-GGT ACA AGA TCT ACT ATA GGG AGA CCG GAA TTC CGC-3', SEQ. ID:11:, and 5'-CCA CAT AGA TCT GTT CCA TGG TTG TGG CAA TAT TAT CAT CG-3', SEQ. ID:15:, respectively. The
 10 mutated residue, underlined in the antisense codon, eliminates an upstream ATG from the preferred ATG contained within the NcoI/Kozak sequence at the 3'-terminal end of the IRES

G) pGEM-3-IRES/REV: HIVIIIb *REV* was PCR amplified from pCV-1 (catalogue #303, NIH AIDS Research and Reference Program)
 15 using synthetic oligomers. The sense and antisense oligomers were 5'-GGT ACA AGA TCT ACC ATG GCA GGA AGA AGC GGA GAC AGC-3', SEQ. ID:16:, and 5'-CCA CAT AGA TCT GAT ATC GCA CTA TTC TTT AGC TCC TGA CTC C-3', SEQ. ID:17:, respectively. These oligomers provide BglII sites at either end of the translation open
 20 reading frame as well as an EcoRV site directly upstream from the BglII site at the 3'-terminal end of *rev*. After PCR, the *REV* gene was treated with NcoI (located within the Kozak sequence) and BglII restriction enzymes and ligated with pGEM-3-IRES which had been treated with NcoI and BamHI restriction enzymes. Each ligation
 25 junction as well as the entire 0.3 kb *REV* gene was confirmed by DNA sequencing.

H) V1Jns-tPA: In order to provide an heterologous leader peptide sequence to secreted and/or membrane proteins, V1Jn was modified to
 30 include the human tissue-specific plasminogen activator (tPA) leader. Two synthetic complementary oligomers were annealed and then ligated into V1Jn which had been BglII digested. The sense and antisense oligomers were 5'-GATC ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT

TCG CCC AGC GA-3', SEQ.ID:18:, and 5'-GAT CTC GCT GGG CGA
AAC GAA GAC TGC TCC ACA CAG CAG CAG CAC ACA GCA
GAG CCC TCT CTT CAT TGC ATC CAT GGT-3', SEQ. ID:19:. The
5 Kozak sequence is underlined in the sense oligomer. These oligomers
have overhanging bases compatible for ligation to BglII-cleaved
sequences. After ligation the upstream BglII site is destroyed while the
downstream BglII is retained for subsequent ligations. Both the
junction sites as well as the entire tPA leader sequence were verified by
10 DNA sequencing. Additionally, in order to conform with our consensus
optimized vector V1Jns (=V1Jneo with an SfiI site), an SfiI restriction
site was placed at the KpnI site within the BGH terminator region of
V1Jn-tPA by blunting the KpnI site with T4 DNA polymerase followed
by ligation with an SfiI linker (catalogue #1138, New England Biolabs).
15 This modification was verified by restriction digestion and agarose gel
electrophoresis.

I) V1Jns-HIVIIIb REV: *REV* was amplified by PCR as described
above for pGEM-3-IRES/*REV*, digested with BglII restriction enzyme,
20 and ligated into V1Jns which had been BglII- and calf intestinal alkaline
phosphatase-treated. Ligation junctions were confirmed by DNA
sequencing and expression of *REV* was verified by *in vitro* transfection
of RD cells and immunoblot analysis (greater than 1 µg *REV* obtained
per 10⁶ cells).

25 J) pGEM-3-RRE/IRES/REV: In order to make a cassette consisting of
the *REV* response element (RRE) which is required to be on an RNA
transcript in order for *REV*-dependent expression to occur, the RRE
from HIV strain HXB2 was obtained by PCR using the following
synthetic oligomers: sense oligomer, 5'-GGT ACA TGA TCA GAT
30 ATC GCCC GGG C CGA GAT CTT CAG ACT TGG AGG AGG AG-
3', SEQ.ID:20:; and antisense oligomer, 5'-CCA CAT TGA TCA G
CTT GTG TAA TTG TTA ATT TCT CTG TCC-3', SEQ.ID:21:.
These oligomers provide BclI restriction sites at either end of the insert
as well as EcoRV and SrfI sites at the 5'-end of the insert. The RRE

was blunt-end ligated into pGEM-3-/IRES/REV at the HincII restriction site which precedes IRES. The ligation products were verified by restriction enzyme mapping and by DNA sequencing across the ligation junctions.

EXAMPLE 2

gp120 Vaccines:

Expression of the *REV*-dependent *env* gene as gp120 was conducted as follows: gp120 was PCR-cloned from the MN strain of HIV with either the native leader peptide sequence (V1Jns-gp120), or as a fusion with the tissue-plasminogen activator (tPA) leader peptide replacing the native leader peptide (V1Jns-tPA-gp120). tPA-gp120 expression has been shown to be *REV*-independent [B.S. Chapman *et al.*, Nuc. Acids Res. **19**, 3979 (1991); it should be noted that other leader sequences would provide a similar function in rendering the gp120 gene *REV* independent]. This was accomplished by preparing the following gp120 constructs utilizing the above described vectors:

I. gp120 VACCINE CONSTRUCTS:

A) V1Jns-tPA-HIVMN gp120: HIVMN gp120 gene (Medimmune) was PCR amplified using oligomers designed to remove the first 30 amino acids of the peptide leader sequence and to facilitate cloning into V1Jns-tPA creating a chimeric protein consisting of the tPA leader peptide followed by the remaining gp120 sequence following amino acid residue 30. This design allows for *REV*-independent gp120 expression and secretion of soluble gp120 from cells harboring this plasmid. The sense and antisense PCR oligomers used were 5'-CCC CGG ATC CTG ATC ACA GAA AAA TTG TGGGTC ACA GTC-3', SEQ. ID:22:, and 5'-C CCC AGG AATC CAC CTG TTA GCG CTT TTC TCT CTG CAC CAC TCT TCT C-3', SEQ. ID:23:. The translation stop codon is underlined. These oligomers contain BamHI restriction enzyme sites at either end of the translation open reading frame with a BclII site located 3' to the BamHI of the sense oligomer. The PCR product was sequentially digested with BclII followed by BamHI and ligated into

V1Jns-tPA which had been BglIII digested followed by calf intestinal alkaline phosphatase treatment. The resulting vector was sequenced to confirm inframe fusion between the tPA leader and gp120 coding sequence, and gp120 expression and secretion was verified by immunoblot analysis of transfected RB cells. Thus, this vector encoding the tPA-HIVMN-gp120 is useful for inclusion in a bi- or tri-cistronic construct expressing gag, B7 or other antigens.

10 B) V1-tPA-HIVMN_gp120: A slightly different version of the chimeric tPA-HIVMN gp120 vector described above was made using an earlier version of our basic vaccine expression vector, V1 (see Nucleic Acid Pharmaceuticals patent), which contained a somewhat different tPA peptide leader sequence from that described for V1Jns-tPA.

15 In either of the foregoing PNV constructs, provision of an IRES sequence after the translation stop codon, and downstream cloning of immunomodulatory genes such as B7, provides bi- or tri-cistronic polynucleotides useful according to the method of this invention. These PNV's efficiently express both gene products.

20 C) V1Jns-tPA-HIVIIIB_gp120: This vector is analogous to I.A. except that the HIV IIIB strain was used for gp120 sequence. The sense and antisense PCR oligomers used were: 5'-GGT ACA TGA TCA CA GAA AAA TTG TGG GTC ACA GTC-3', SEQ.ID:24:, and 5'-CCA CAT TGA TCA GAT ATC TTA TCT TTT TTC TCT CTG CAC CAC TCT TC-3', SEQ.ID:25:, respectively. These oligomers provide BclI sites at either end of the insert as well as an EcoRV just upstream of the BclI site at the 3'-end. The 5'-terminal BclI site allows ligation into the BglII site of V1Jns-tPA to create a chimeric tPA-gp120 gene encoding the tPA leader sequence and gp120 without its native leader sequence. Ligation products were verified by restriction digestion and DNA sequencing.

II. IN VITRO gp120 VACCINE EXPRESSION:

5 In vitro expression was tested in transfected human rhabdomyosarcoma (RD) cells for these constructs. Quantitation of secreted tPA-gp120 from transfected RD cells showed that V1Jns-tPA-gp120 vector produced secreted gp120.

III. IN VIVO gp120 VACCINATION:

10 See figure 12 (mouse data):

Anti-gp120 ELISA Titers Elicited by Secreted gp120*

15

<u>Species</u>	<u>GMT (range)</u>
mouse (post 2 rounds, 200µg per round)	5,310 (1.8×10^3 - 1.5×10^4)
rabbit (post 3 rounds, 2 mg per round)	143 (75- 212)
A.G. monkey (post 2 rounds, 2 mg per round)	171 (<10-420)

30 *Using V1Jns-tPA-gp120III B as the inoculation vector, intramuscularly.

V1Jns-tPA-gp120MN PNV-induced Class II MHC-
restricted T lymphocyte gp120 specific antigen reactivities. Balb/c mice
which had been vaccinated two times with 200 µg V1Jns-tPA-gp120MN
were sacrificed and their spleens extracted for in vitro determinations
5 of helper T lymphocyte reactivities to recombinant gp120. T cell
proliferation assays were performed with PBMC (peripheral blood
mononuclear cells) using recombinant gp120IIIB (Repligen, catalogue
#RP1016-20) at 5 µg/ml with 4×10^5 cells/ml. Basal levels of ^3H -
10 thymidine uptake by these cells were obtained by culturing the cells in
media alone, while maximum proliferation was induced using ConA
stimulation at 2 µg/ml. ConA-induced reactivities peak at ~3 days and
were harvested at that time point with media control samples while
antigen-treated samples were harvested at 5 days with an additional
15 media control. Vaccinated mice responses were compared with naive,
age-matched syngenic mice. ConA positive controls gave very high
proliferation for both naive and immunized mice as expected. Very
strong helper T cell memory responses were obtained by gp120
treatment in vaccinated mice while the naive mice did not respond (the
20 threshold for specific reactivity is an stimulation index (SI) of >3-4; SI
is calculated as the ratio of sample cpm/media cpm). SI's of 65 and 14
were obtained for the vaccinated mice which compares with anti-gp120
ELISA titers of 5643 and 11,900, respectively, for these mice.
Interestingly, for these two mice the higher responder for antibody gave
25 significantly lower T cell reactivity than the mouse having the lower
antibody titer. This experiment demonstrates that the secreted gp120
vector efficiently activates helper T cells in vivo as well as generates
strong antibody responses. In addition, each of these immune responses
was determined using antigen which was heterologous compared to that
30 encoded by the inoculation PNV (IIIB vs. MN):

Splenic T Cell Proliferation Responses to rgp120 Following Vaccination with V1Jns-tPA-gp120MN

Avg. CPM (Stimulation Index)

5	Mouse # (agp120 titer) ³	Media ¹	ConA ¹	Media ²
	rgp120 ²			
	#1 (naive; <10) 339 (1)	185,358 (546)	187 (1)	574 (3)
10	#2 (naive; <10) 237 (1)	229,775 (969)	283 (1)	511 (1.8)
	#3 (immune; 5643) 317 (1)	221,003 (697)	354 (1)	23,109 (65)
15	#4 (immune; 11,900) 229 (1)	243,427 (1063)	235 (1)	3384 (14)

¹Cells harvested on day 4 following 24 hr with ³H-thymidine. ConA was used at 2 µg/ml concentration.

²Cells harvested on day 5 following 24 hr with ³H-thymidine. Recombinant gp120_{III}B was used at 5 µg/ml concentration.

³Anti-gp120_{III}B reciprocal endpoint ELISA titers and proliferation assays performed following 2 rounds of 200 µg DNA/mouse (Balb/c).

The foregoing data clearly demonstrates efficient in vivo expression of relevant HIV antigens with a polynucleotide vaccine antigen and elicitation of specific immune responses to the expressed gene product. This construct is easily modified to form a bi-cistronic PNV of this invention by including, downstream from the gp120 translation stop codon, an second or third cistron encoding REV, B7, gag or other antigens unrelated to HIV, such as influenza nucleoprotein or hemagglutinin encoding genes.

EXAMPLE 3

gp160 VACCINES

5 In addition to secreted gp120 constructs, we have prepared expression constructs for full-length, membrane-bound gp160. The rationales for a gp160 construct, in addition to gp120, are (1) more epitopes are available both for both CTL stimulation as well as neutralizing antibody production including gp41, against which a potent HIV neutralizing monoclonal antibody (2F5, see above) is directed; (2) a more native protein structure may be obtained relative to virus-
10 produced gp160; and, (3) the success of membrane-bound influenza HA constructs for immunogenicity [Ulmer et al., *Science* **259**:1745-1749, 1993; Montgomery, D., et al., *DNA and Cell Biol.* **12**:777-783, 1993].

gp160 retains substantial *REV* dependence even with a heterologous leader peptide sequence. Therefore, two strategies
15 independent from that employed for gp120 expression were developed for preparing a gp160 expression vector: (1) subcloning into V1Jns a genomic HIV DNA fragment reported to be effective for heterologous gp160 expression containing *tat*, *REV* and gp160 in entirety (V1Jns-*tat/REV/env*), [Wang et al., *P.N.A.S. USA* **90**:4156-4160 (May, 1993);
20 all of the data reported in that study were generated using bupivacaine injection about 24 hours prior to nucleic acid injection. As bupivacaine is known to cause muscle damage, this is a regiment that clearly could not be used to immunize humans], and (2) PCR-cloning a minimal gp160 ORF into a dicistronic vector before the EMCV internal
25 ribosomal entry site (IRES) to efficiently reinitiate translation following gp160 translation for a second cistron encoding *REV*. This construct ensures effective simultaneous production of both gp160 and *REV* proteins (V1Jns-gp160/IRES/*rev*). Each of these vectors has been prepared in addition to the monocistronic vectors V1Jns-gp160 and
30 V1Jns-*REV*. Because there is evidence, in the literature and from our own experiments (see below), that the *env* mRNA requires the *tat/REV* splice donor (SD) site for stability in heterologous expression systems, V1Jns-gp160 and V1Jns-gp160/IRES/*REV* were also prepared with this

SD inserted upstream of the *env* ORF. These vaccine constructs were prepared as follows.

I. gp160 VACCINE CONSTRUCTS:

5 Both gp160 expression vectors, V1Jns-gp160 and V1Jns-gp160/IRES/rev (see A and B below) were prepared with the tat/rev splice donor (SD) inserted immediately upstream of gp160 sequences at the PstI site within V1Jns (this is the solitary PstI site within both of these vectors). Synthetic complementary oligomers encoding the SD
10 were designed to ligate into the PstI site retaining the original site at the 5'-end but destroying the PstI site at the 3'-end of the insert after ligation. The oligomer sequences used were: 5'-GTC ACC GTC CTC TAT CAA AGC AGT AAG TAG TAC ATG CA-3', SEQ.ID:26; and 5'-TGT ACT ACT TAC TGC TTT GAT AGA GGA CGG TGA CTG
15 CA-3', SEQ.ID:27;. The resulting plasmids were verified by restriction digestion mapping and by DNA sequencing across the entire SD/PstI region.

20 A). V1Jns-HIVIII_bgp160: HIVIII_b gp160 was cloned by PCR amplification from plasmid pF412 which contains the 3'-terminal half of the HIVIII_b genome derived from HIVIII_b clone HXB2. The PCR sense and antisense oligomers were 5'-GGT ACA TGA TCA ACC ATG AGA GTG AAG GAG AAA TAT CAG C-3', SEQ. ID:28;, and 5'-CCA CAT TGA TCA GAT ATC CCC ATC TTA TAG CAA AAT CCT TTC
25 C-3', SEQ. ID:29;, respectively. The Kozak sequence and translation stop codon are underlined. These oligomers provide BclI restriction enzyme sites outside of the translation open reading frame at both ends of the *env* gene. (BclI-digested sites are compatible for ligation with BglII-digested sites with subsequent loss of sensitivity to both restriction
30 enzymes. BclI was chosen for PCR-cloning gp160 because this gene contains internal BglII and as well as BamHI sites). The antisense oligomer also inserts an EcoRV site just prior to the BclI site as described above for other PCR-derived genes. The amplified gp160 gene was agarose gel-purified, digested with BclI, and ligated to V1Jns

which had been digested with BglII and treated with calf intestinal alkaline phosphatase. The cloned gene was about 2.6 kb in size and each junction of gp160 with V1Jns was confirmed by DNA sequencing.

5 B). V1Jns-HIVIII_b gp160/IRES/REV: pGEM-3-IRES/REV was digested with HindII and SmaI restriction enzymes (contained within the pGEM-3 multi-linker region) to remove the entire IRES/REV sequence (~0.9 kb) and then ligated with V1Jns-HIVIII_bgp160 which
10 had been digested with EcoRV and phosphatased. This procedure yielded an 8.3 kb dicistronic V1Jns containing gp160 followed by IRES and REV which directs expression of both of these HIV gene products. All of the junction regions were verified by DNA sequencing.

15 C) V1Jns-tPA-HIVIII_B gp160: This vector is similar to Example 2(C) above, except that the full-length gp160, without the native leader sequence, was obtained by PCR. The sense oligomer was the same as used in I.C. and the antisense oligomer was 5'-CCA CAT TGA TCA GAT ATC CCC ATC TTA TAG CAA AAT CCT TTC C-3',
20 SEQ.ID:30:. These oligomers provide BclI sites at either end of the insert as well as an EcoRV just upstream of the BclI site at the 3'-end. The 5'-terminal BclI site allows ligation into the BglII site of V1Jns-tPA to create a chimeric tPA-gp160 gene encoding the tPA leader sequence and gp160 without its native leader sequence. Ligation products were
25 verified by restriction digestion and DNA sequencing.

30 D) V1Jns-tat/rev/envIII_B: This expression vector is patterned after one described by D. Rekosh et al. [Proc. Natl. Acad. Sci. USA, 85, 334 (1988)] employing a "genomic" segment of an HIV-1 III_B clone (HXB2) encompassing unspliced *tat*, *rev*, and *env* in their entirety. V1Jns was
30 digested with BglII followed by T4 DNA polymerase blunting and calf intestinal alkaline phosphatase treatment. A Sall/XhoI fragment of the III_B genome contained within pF412 was obtained by restriction digestion and blunted with T4 DNA polymerase. Ligation products were verified by restriction digestion mapping and DNA sequencing.

E) V1Jns-rev/envIIIB: This vector is a variation of the one described in section D above except that the entire *tat* coding region in exon 1 is deleted up to the beginning of the *REV* open reading frame. V1Jns-gp160III B (see section A. above) was digested with PstI and KpnI restriction enzymes to remove the 5'-region of the gp160 gene. PCR amplification was used to obtain a DNA segment encoding the first *REV* exon up to the KpnI site in gp160 from the HXB2 genomic clone. The sense and antisense PCR oligomers were 5'-GGT ACA CTG CAG TCA CCG TCC T ATG GCA GGA AGA AGC GGA GAC-3', SEQ.ID:31: and 5'-CCA CAT CA GGT ACC CCA TAA TAG ACT GTG ACC-3', SEQ.ID:32: respectively. These oligomers provide PstI and KpnI restriction enzyme sites at the 5'- and 3'- termini of the DNA fragment, respectively. The resulting DNA was digested with PstI and KpnI, purified from an agarose electrophoretic gel, and ligated with V1Jns-gp160(PstI/KpnI). The resulting plasmid was verified by restriction enzyme digestion.

II. IN VITRO EXPRESSION OF gp160 VACCINE:

RD and 293 cells were transiently transfected with gp160 and REV expression constructs. A Western blot analysis shown in Fig. 5 using an anti-gp41 monoclonal antibody (Chessie 8, NIH AIDS Research and Reference Program #526) showed that gp160 expression by V1Jns-gp160 (SD) required the addition of V1Jns-REV (this vector produces $> 1 \mu\text{g REV}/10^6$ cells in transient transfections). V1Jns-gp160/IRES/REV efficiently expressed gp160 without additional REV added in trans, confirming function of the dicistron. Similar results were found with an anti-gp120 monoclonal antibody (1C1, Repligen, #RP1010-10) for immunoblot visualization. Proteolytic processing of gp160 to the mature gp120 and gp41 forms was observed for each vector. Addition of REV in trans to the dicistronic gp160/REV vector did not result in more gp160 expression indicating that REV expression is not limiting for gp160 expression in this vector. Expression of gp160 improved if the *tat*/REV SD was included within dicistronic gp160/REV

construct indicating the importance of this site for optimal REV-dependent gp160 expression. We were also surprised to discover that dicistronic gp160/REV expressed more than ten-fold more gp160 than the genomic tat/REV/env construct for transient transfections, again demonstrating the high efficiency of this vector for gp160 expression.

These vectors provide nucleic acid constructs for gp160 plasmid vaccinations with gp160 and *REV* genes either on separate plasmids or on the same plasmid. In the case of the tpa-gp160 construct, *REV* need not be provided in cis or in trans to achieve efficient gp160 expression, therefore allowing other genes to be incorporated in a dicistronic construct.

For the REV-dependent constructs, it is important to test whether effective gp160 expression following vaccination requires *REV* to be present on the same plasmid because very small quantities of DNA are taken up by muscle cells following intramuscular injection, and individual muscle cells (each having hundreds of nuclei) may not receive copies of different plasmids in proximal locations within the cell.

III. IN VIVO VACCINATION WITH gp160 VACCINES:

Three different vector strategies were compared for their abilities to induce anti-gp120 antibody responses in nonhuman primates using PNVs encoding gp160: vaccination with (1) dicistronic gp160/REV using V1Jns-gp160III_B/IRES/REV (SD); (2) the genomic gp160 construct V1Jns/tat/rev/envIII_B; and (3) a mixture of monocistronic vectors, V1Jns-gp160III_B (SD) and V1Jns-REV.

Vaccination doses of 2 mg/animal were used for up to three vaccination rounds which were delivered at one month intervals while simultaneously obtaining bleeds. Anti-gp120 ELISA titers using recombinant gp120III_B are shown for monkeys vaccinated with each of these vectors. Dicistronic gp160/REV elicited antibody responses in both rhesus and African Green monkeys while the genomic gp160 and mixed monocistronic vectors did not elicit detectable antibodies after two rounds of vaccination (i.e., one month following the second

vaccination). All four monkeys which received dicistronic gp160/REV also showed specific anti-gp41 reactivities as measured by the BIAcore assay using recombinant gp41 (ABT) as the immobilized substrate (data not shown). The sera obtained from these monkeys also showed anti-V3III_B ELISA reactivities with titers ranging from ~50 - 100. These results prove that in vivo expression induced by PNV for multiple cistrons is not analogous to results obtained by in vitro transfection methods in which gp160 expression was shown for all three vector strategies. Note especially that in vitro transfection resulted in equivalent expression by the mixed monocistronic gp160 and REV vectors as compared to dicistronic gp160/REV (see Fig. 5). These experiments prove that our dicistronic PNVs do deliver effective coordinate expression following in vivo vaccination while other methods of vaccination with multiple cistrons were unable to do so. See figure 9, showing two African Green Monkeys and two rhesus monkeys and one rabbit's immune responses.

Anti-gp120 ELISA Titers Elicited by gp160 PNVs
in Non-Human Primates
2 mg DNA per round

5

<u>Vector/Species</u>		<u>Titer</u>	
		<u>post 2nd</u>	<u>post 3rd</u>
<u>V1Jns-tat/rev/env_{III}B:</u>			
10	African Green (#1)	<20	ND ¹
	(#2)	<20	ND
	(#3)	<20	ND
<u>V1Jns-gp160_{III}B + V1Jns-rev²:</u>			
15	African Green (#1)	<20	ND
	(#2)	<20	ND
	(#3)	<20	ND
<u>V1Jns-gp160_{III}B/IRES/rev :</u>			
20	African Green (#1)	85	90
	(#2)	75	60
	Rhesus (#1)	165	175
	(#2)	290	260

25

¹ND = not determined.

²This PNV represents an equimolar mixture of the two monocistronic vectors.

30

Anti-V3_{III B} ELISA Titers Elicited by gp160/rev Dicistron*
in Non-Human Primates. 2 mg DNA per round

	<u>Species (animal #)</u>	<u>Titer (post 3rd vaccination)</u>
5		
	African Green (#1)	70
	(#2)	45
10	Rhesus (#1)	55
	(#2)	100

*Using V1Jns-gp160_{III B}/IRES/rev as the inoculation vector.

15

EXAMPLE 4

SIV VACCINES

20

25

An SIV *env* construct, V1Jn-SIV gp152, was made by PCR-cloning from a genomic clone of the SIVMAC251 virus isolate and confirmed by DNA sequencing of both junctions with the vector. This strain is homologous to the virus which is used at the New England Regional Primate Center (NRPC) for infectious SIV challenges to rhesus monkeys. A similar SIV gp152 construct is prepared in which the DNA encoding the leader peptide region uses alternative codons but which retains the native amino acid sequence. This reduces the *REV*-dependence of this construct and makes a more stable mRNA transcript. These vaccine constructs were prepared as follows.

I. SIV VACCINE CONSTRUCTS:

30

A). V1J-SIVMAC251 p28 *gag* : The central peptide of SIV *gag* , referred to as p28 *gag* , was chosen for a polynucleotide vaccine to test for CTL generation in nonhuman primates. This region of *gag* encodes a known CTL epitope for macaque monkeys which have the MHC Class I haplotype known as Mamu-A01. Thus, monkeys bearing this haplotype should demonstrate CTL reactivity this *gag*

epitope after vaccination with the appropriate *gag* plasmid. While both SIV and HIV *gag* genes contain regulatory sequences which are *REV* dependent, p28 *gag* expression appears to be less *REV* -dependent so that at least some expression may be achieved in the absence of *REV* .

5 SIV p28 *gag* was cloned into expression vectors V1 using BglII restriction enzyme sites after PCR amplification from the plasmid p239SpSp5' (obtained from the NIH AIDS Research and Reference Program, catalogue #829) using custom synthetic
10 oligodeoxyribonucleotides. This plasmid encodes the 5'- half of the SIVMAC239 genome. SIVMAC 239 is a subsequent in vitro passage line of SIVMAC251 which has undergone some mutations compared to the parental virus. However, the amino acid sequences between these viruses are identical for p28 *gag*. The PCR sense and antisense
15 oligomers were 5'-GGT ACA AGA TCT ACC ATG GGA CCA GTA CAA CAA ATA GGT GGT AAC-3' , SEQ. ID:33:, and 5'-CCA CAT AGA TCT TTA CAT TAA TCT AGC CTT CTG TCC C-3', SEQ. ID:34:.. These oligos provide BglII restriction enzyme sites outside the translational open frames, a consensus Kozak translation initiation codon context (underlined) and translation stop codon (underlined). PCR-
20 generated p28 *gag* was agarose gel-purified, digested with BglII and ligated into BglII-treated, phosphatased V1. This gene was subsequently subcloned into our optimized expression vector, V1J, using BglII restriction enzyme sites and designated as V1J-SIV p28 *gag*. The cloned gene was about 0.7 kb long. The junction sites of the V1J CMV
25 promoter and 5'terminus of p28 *gag* were verified by DNA sequence analysis for each construct. In vitro expression of SIV p28 protein was compared for V1J and V1 constructs by Western blotting using plasma from an SIV-infected macaque monkey to detect *gag* protein. The V1J-SIV p28 *gag* construct consistently gave the most product at the
30 appropriate molecular weight position. Similar and even improved results are obtained with the more optimized V1jneo, V1Jns and V1R vectors.

B). V1J-SIVMAC251 *nef*: SIV *nef* was cloned after PCR amplification from the plasmid pBK28 which encodes the entire SIVMAC251 genome (a gift from Dr. Vanessa Hirsch, NIAID, NIH, Rockville, MD; now listed as catalogue #133, NIH AIDS Research and Reference Program). The PCR sense and antisense oligomers were 5'-GGT ACA ACC ATG GGT GGA GCT ATT TCC ATG AGG-3', SEQ. ID:35: and 5'-CCT AGG TTA GCC TTC TTC TAA CCT CTT CC-3', SEQ. ID:36:. The Kozak site and translation stop codon are underlined. The amplified *nef* gene was agarose gel-purified, blunt-ended using T4 DNA polymerase, phosphorylated at the 5'-terminus using T4 DNA kinase, and cloned into a blunted BglII restriction enzyme site of V1J which had been phosphatased using calf intestinal alkaline phosphatase. The cloned gene was about 0.76 kb long. The junction site of the V1J CMV promoter and 5'-terminus of *nef* was confirmed by DNA sequencing. In vitro expression was shown using Western blot analysis and an HIV *nef* antiserum (catalogue #331, NIH AIDS Research and Reference Program).

C). V1Jn-SIVMAC251 gp152: SIV *env*, referred to as gp152, was cloned after PCR amplification from the plasmid pBK28 into V1Jneo (see Nucleic Acid Pharmaceuticals patent). The PCR sense and antisense oligomers were 5'-GGT ACA AGA TCT ACC ATG GGA TGT CTT GGG AAT CAG C-3', SEQ. ID:37: and 5'-CCA CAT AGA TCT GAT ATC GTA TGA GTC TAC TGG AAA TAA GAG G-3', SEQ.ID:38. The Kozak site and *amber* translation stop codon are underlined. The PCR product has BglII restriction enzyme sites outside the translation open reading frame at both ends with an additional EcoRV site immediately preceding the 3'-terminal BglII site but after the *amber* stop codon. This provides a convenient restriction enzyme site for subsequent cloning steps. The amplified gp152 gene was agarose gel-purified, BglII-digested and ligated with V1Jn which had been BglII-digested and phosphatased. The cloned gene was about 2.2 kb long. The junctions at each end of gp152 with V1Jn CMV promoter and BGH terminator regions were verified by DNA sequencing.

II. IN VIVO VACCINATION WITH SIV VACCINES:

Two SIV gene constructs have been used for vaccination of rhesus monkeys and have been shown to generate specific CTL responses in non-human primates (see figure 4).

V1J-SIV p28 *gag*, which expresses the relatively *REV*-independent central peptide of *gag*, and V1J-SIV *nef* were i.m.-injected into *Macaca mulatta* monkeys at 3mg/vaccination for three injection rounds spaced one month apart. The *gag*-specific CTL response of rhesus monkeys with the Mamu-A01 MHC I haplotype is restricted primarily to a single peptide epitope within p28 *gag*. Mamu-A01⁺ monkeys receiving V1J-SIV p28 *gag* had *gag*-specific CTL activity beginning at one month after the second injection while Mamu-A01⁻ monkeys receiving this DNA as well as monkeys receiving V1J control DNA did not show a CTL response. Both in vitro, *gag* peptide-restimulated CTL as well as primary CTL were detected after the second and third vaccination rounds, respectively. These CTL activity levels were comparable to those generated by vaccinia-*gag* inoculation. Subsequently, the CTL levels declined in responding animals. These animals are re-vaccinated to boost the initial CTL response. V1J-SIV *nef*-vaccinated animals have not shown a specific CTL response, although a more refined assay, such as the one used for *gag* CTL detection, (i.e., no dominant MHC I haplotype/*nef* peptide relationship has been defined for rhesus monkeys so that peptides of unknown effectiveness are used for stimulation, and there is no positive control), may provide a different result.

EXAMPLE 5

OTHER VACCINE CONSTRUCTS

A. V1Jns-HIV_{IIIB} *gag/pol*-RRE/IRES/REV: Dicistronic expression vectors encoding *gag* with or without the *protease* region of *pol* were made by PCR amplification of HIV_{IIIB} *gag-pol* sequences with several variations. Inclusion of the *protease* (*prt*) segment of *pol* allows proteolytic processing of *gag* into various peptides (e.g., p17,

p24, p15, etc.) which comprise the mature capsid particles while omission of this enzyme results in p55 synthesis in the form of immature capsid particles. More extensive sequences of *pol* were not included to avoid potential safety hazards that may be associated with the reverse transcriptase and integrase enzymatic activities of *pol*. For *gag* capsid particles, whether processed into the mature forms or not, to be extruded from cells myristoylation of the glycine amino acid at position two following the initial Met must occur. Mutagenesis of this glycine residue abrogates myristoylation and no *gag* particles are extruded from the cell. These modifications of *gag* allow us to determine whether either generation of anti-*gag* CTLs following vaccination with such *gag* vectors is affected by proteolytic processing and/or extrusion of capsid from cells. Some of the vectors listed below contain a splice donor (SD) site that is found upstream of the *gag* open reading frame. These vectors allow us to determine whether this SD is necessary for optimum *rev*-dependent expression of *gag* as was inclusion of the *tat/rev* SD for optimum gp160 expression.

1) V1Jns-HIVIIIB *gag-prt*/RRE/IRES/REV: A *gag-prt* encoding DNA segment was obtained by PCR amplification using the following sense and antisense oligomers: 5'-GGT ACA GGA TCC ACC ATG GGT GCG AGA GCG TCA GTA TTA AGC-3', SEQ.ID:39; and 5'-CCA CAT GGA TCC GC CCG GGC TTA CAT CTC TGT ACA AAT TTC TAC TAA TGC-3', SEQ.ID:40; respectively. These oligomers provide BamHI restriction enzyme sites at either end of the segment, a Kozak initiation of translation sequence including an NcoI site, and an SrfI site immediately upstream of the BamHI site at the 3'-terminus. The SrfI site was used to clone the RRE/IRES/REV cassette from pGEM-3-RRE/IRES/REV, which was excised using the EcoRV restriction enzyme, immediately downstream of *gag-prt*. All ligation junctions were DNA sequence verified and the construct was further verified by restriction enzyme mapping.

2) V1Jns-HIVIIIB *gag-prt*/RRE/IRES/REV(SD): This vector was prepared exactly as vector 1 above except that the PCR sense oligomer used was 5'-GGT ACA GGA TCC CCG CAC GGC AAG

AGG CGA GGG-3', SEQ.ID:41:. This allows inclusion of the upstream SD site at the beginning of the *gag* sequence. This construct was verified by restriction enzyme mapping and DNA sequencing of the ligation junctions.

5 3) V1Jns-HIV_{IIIB} *gag*-*prt*/RRE/IRES/REV(w/o myristoylation): This vector is prepared exactly as vector 1 above except that the PCR sense oligomer used was 5'-GGT ACA GGA TCC ACC ATG GCT GCG AGA GCG TCA GTA TTA AGC-3', SEQ.ID:42.

10 4) V1Jns-HIV_{IIIB} *gag*/RRE/IRES/REV: This vector is prepared exactly as vector 1 above except that the PCR antisense oligomer used was 5'-CCA CAT GGA TCC GCC CGG GCC TTT ATT GTG ACG AGG GGT CGT TGC-3', SEQ.ID:43.

15 5) V1Jns-HIV_{IIIB} *gag*/RRE/IRES/REV (SD): This vector is prepared exactly as vector 4 above except that the PCR sense oligomer used was 5'-GGT ACA GGA TCC CCG CAC GGC AAG AGG CGA GGG-3', SEQ.ID:44.

20 6) V1Jns-HIV_{IIIB} *gag*/RRE/IRES/REV (w/o myristoylation): This vector is prepared exactly like vector 5 except that the PCR sense oligomer used was 5'-GGT ACA GGA TCC ACC ATG GCT GCG AGA GCG TCA GTA TTA AGC-3', SEQ.ID:45.

25 B. V1Jns-HIV *nef*: This vector uses a *nef* gene from a viral strain representative of those in the infected population using sense and antisense PCR oligomers analogous to those used for SIV *nef*.

30 C. pGEM-3-X-IRES-B7: (where X = any antigenic gene)
As an example of a dicistronic vaccine construct which provides coordinate expression of a gene encoding an immunogen and a gene encoding an immunostimulatory protein, the murine B7 gene was PCR amplified from the B lymphoma cell line CH1 (obtained from the ATCC). B7 is a member of a family of proteins which provide essential costimulation T cell activation by antigen in the context of major histocompatibility complexes I and II. CH1 cells provide a good source of B7 mRNA because they have the phenotype of being constitutively

activated and B7 is expressed primarily by activated antigen presenting cells such as B cells and macrophages. These cells were further stimulated in vitro using cAMP or IL-4 and mRNA prepared using standard guanidinium thiocyanate procedures. cDNA synthesis was performed using this mRNA using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus) and a priming oligomer (5'-GTA CCT CAT GAG CCA CAT AAT ACC ATG-3', SEQ.ID:46:) specific for B7 located downstream of the B7 translational open reading frame. B7 was amplified by PCR using the following sense and antisense PCR oligomers: 5'-GGT ACA AGA TCT ACC ATG GCT TGC AAT TGT CAG TTG ATG C-3', SEQ.ID:47:, and 5'-CCA CAT AGA TCT CCA TGG GAA CTA AAG GAA GAC GGT CTG TTC-3', SEQ.ID:48:, respectively. These oligomers provide BglII restriction enzyme sites at the ends of the insert as well as a Kozak translation initiation sequence containing an NcoI restriction site and an additional NcoI site located immediately prior to the 3'-terminal BglII site. NcoI digestion yielded a fragment suitable for cloning into pGEM-3-IRES which had been digested with NcoI. The resulting vector, pGEM-3-IRES-B7, contains an IRES-B7 cassette which can easily be transferred to V1Ins-X, where X represents an antigen-encoding gene.

D. pGEM-3-X-IRES-GM-CSF: (where X = any antigenic gene) This vector contains a cassette analogous to that described in item C above except that the gene for the immunostimulatory cytokine, GM-CSF, is used rather than B7. GM-CSF is a macrophage differentiation and stimulation cytokine which has been shown to elicit potent anti-tumor T cell activities in vivo [G. Dranoff et al., Proc. Natl. Acad. Sci. USA, 90, 3539 (1993)].

E. pGEM-3-X-IRES-IL-12: (where X = any antigenic gene) This vector contains a cassette analogous to that described in item C above except that the gene for the immunostimulatory cytokine, IL-12, is used rather than B7. IL-12 has been demonstrated to have an influential role in shifting immune responses towards cellular, T cell-

dominated pathways as opposed to humoral responses [L. Alfonso et al., Science, 263, 235, 1994].

5 F. V1Jns-HIV_x gp160/IRES/revIIIB (SD): This vector is analogous to the one described in I.B. above except that gp160 genes derived from various clinical strains are used rather than gp160 derived from laboratory strain IIIB.

10 G. V1Jns-PR8/34/HA-IRES-SIV p28 gag

This construct provides an influenza hemagglutination gene (HA) in concert with the SIV p28 gag gene for coordinate expression via the IRES element. The PR8/34/HA gene was amplified by PCR using the following sense and antisense oligomers: 5'-GGT ACA AGA TCT ACC ATG AAG GCA AAC CTA CTG GTC CTG-3',
15 SEQ.ID:49:, and 5'-CCA CAT AGA TCT GAT ATC CTA ATC TCA GAT GCA TAT TCT GCA CTG C-3', SEQ.ID:50:, respectively. The resulting DNA segment has BglII restriction enzyme sites at either end and an EcoRV site at the 3'-terminus. After BglII digestion this gene was cloned into V1Jns which had been digested with BglII followed by alkaline phosphatase treatment. SIV p28 gag was excised from V1J-SIV p28 gag by NcoI and BglII digestion. pGEM-IRES was digested with NcoI and BamHI for directional ligation with p28 gag/NcoI/BglII. The IRES-p28 gag cassette is removed by restriction digestion with SmaI and HindII and ligated into the EcoRV site of V1Jns-A/PR8/HA. In
20 vivo coordinate expression of these genes allows generation of potent antibody responses by PNV vaccination (HA), with requisite T cell help, which provides such help in a local environment to potentiate the CTL response of the second gene product (SIV p28 gag). This construct also demonstrates the ability to use the PNV and method of this invention to
25 generate immune responses against multiple antigens whether or not related to HIV. Those skilled in the art will appreciate that this type of construct could be mixed with other, bi- or tri-cistronic constructs to produce a multivalent combination polynucleotide vaccine.
30

H. V1Jns-tPA-gp160III_B/IRES/SIV p28 gag : V1Jns-tPA-gp160III_B was digested with EcoRV, treated with calf intestinal alkaline phosphatase, and ligated with IRES-SIV p28 gag which had been removed from pGEM-3-IRES-SIV p28 by restriction enzyme digestion using SmaI and HindIII. In vivo coordinate expression of these genes allows coupling a protein which generates strong helper T cell responses (gp160) to one which provides Class I MHC-associated CTL epitopes (SIV p28 gag). This vaccine is designed for immunization of rhesus monkeys for generation of anti-*env* neutralizing antibodies and CTL as well as anti-SIV gag CTL. These monkeys are subsequently challenged with appropriate SHIV viral challenges [see J. Li et al., J. A.I.D.S. 5, 639-646 (1992)].

I. V1Jns-tPA-gp120III_B/IRES/SIV p28 gag : This vector is constructed exactly as V1Jns-tPA-gp160III_B/IRES/SIV p28 gag except that V1Jns-tPA-gp120III_B is used in place of the gp160 gene. Vaccination and SHIV challenge are conducted as described above.

J. V1Jns-tPA-gp120III_B/IRES/HIV gag /IRES/rev: This vector is similar to those described above except that a tricistron provides gag and rev expression in addition to gp120.

K. V1Jns-tPA-gp160III_B/IRES/HIV gag /IRES/rev: This vector is similar to those described above except that a tricistron provides gag and rev expression in addition to gp160.

EXAMPLE 6

ASSAY FOR HIV CYTOTOXIC T-LYMPHOCYTES:

The methods described in this section illustrate the assay as used for vaccinated mice. An essentially similar assay can be used with primates except that autologous B cell lines must be established for use as target cells for each animal. This can be accomplished for humans using the Epstein-Barr virus and for rhesus monkey using the herpes B virus.

Peripheral blood mononuclear cells (PBMC) are derived from either freshly drawn blood or spleen using Ficoll-Hypaque centrifugation to separate erythrocytes from white blood cells. For mice, lymph nodes may be used as well. Effector CTLs may be prepared from the PBMC either by in vitro culture in IL-2 (20 U/ml) and concanavalin A (2µg/ml) for 6-12 days or by using specific antigen using an equal number of irradiated antigen presenting cells. Specific antigen can consist of either synthetic peptides (9-15 amino acids usually) that are known epitopes for CTL recognition for the MHC haplotype of the animals used, or vaccinia virus constructs engineered to express appropriate antigen. Target cells may be either syngenic or MHC haplotype-matched cell lines which have been treated to present appropriate antigen as described for in vitro stimulation of the CTLs. For Balb/c mice the P18 peptide (ArgIleHisIleGlyProGlyArgAlaPheTyrThrThrLysAsn, SEQ.ID:51:, for HIV MN strain) can be used at 10 µM concentration to restimulate CTL in vitro using irradiated syngenic splenocytes and can be used to sensitize target cells during the cytotoxicity assay at 1-10 µM by incubation at 37°C for about two hours prior to the assay. For these H-2^d MHC haplotype mice, the murine mastocytoma cell line, P815, provides good target cells. Antigen-sensitized target cells are loaded with Na⁵¹CrO₄, which is released from the interior of the target cells upon killing by CTL, by incubation of targets for 1-2 hours at 37°C (0.2 mCi for ~5 x 10⁶ cells) followed by several washings of the target cells. CTL populations are mixed with target cells at varying ratios of effectors to targets such as 100:1, 50:1, 25:1, etc., pelleted together, and incubated 4-6 hours at 37°C before harvest of the supernatants which are then assayed for release of radioactivity using a gamma counter. Cytotoxicity is calculated as a percentage of total releasable counts from the target cells (obtained using 0.2% Triton X-100 treatment) from which spontaneous release from target cells has been subtracted.

EXAMPLE 7

ASSAY FOR HIV SPECIFIC ANTIBODIES:

ELISAs were designed to detect antibodies generated against HIV using either specific recombinant protein or synthetic peptides as substrate antigens. 96 well microtiter plates were coated at 4°C overnight with recombinant antigen at 2 µg/ml in PBS (phosphate buffered saline) solution using 50 µl/well on a rocking platform. Antigens consisted of either recombinant protein (gp120, rev: Repligen Corp.; gp160, gp41: American Bio-Technologies, Inc.) or synthetic peptide (V3 peptide corresponding to virus isolate sequences from IIIB, etc.: American Bio-Technologies, Inc.; gp41 epitope for monoclonal antibody 2F5). Plates were rinsed four times using wash buffer (PBS/0.05% Tween 20) followed by addition of 200µl/well of blocking buffer (1% Carnation milk solution in PBS/0.05% Tween-20) for 1 hr at room temperature with rocking. Pre-sera and immune sera were diluted in blocking buffer at the desired range of dilutions and 100 µl added per well. Plates were incubated for 1 hr at room temperature with rocking and then washed four times with wash buffer. Secondary antibodies conjugated with horse radish peroxidase, (anti-rhesus Ig, Southern Biotechnology Associates; anti- mouse and anti-rabbit Igs, Jackson Immuno Research) diluted 1:2000 in blocking buffer, were then added to each sample at 100 µl/well and incubated 1 hr at room temperature with rocking. Plates were washed 4 times with wash buffer and then developed by addition of 100 µl/well of an o-phenylenediamine (o-PD, Calbiochem) solution at 1 mg/ml in 100 mM citrate buffer at pH 4.5. Plates were read for absorbance at 450 nm both kinetically (first ten minutes of reaction) and at 10 and 30 minute endpoints (Thermo-max microplate reader, Molecular Devices).

EXAMPLE 8

ASSAY FOR HIV NEUTRALIZING ANTIBODIES:

In vitro neutralization of HIV isolates assays using sera derived from vaccinated animals was performed as follows. Test sera and pre-immune sera were heat inactivated at 56°C for 60 min before

use. A titrated amount of HIV-1 was added in 1:2 serial dilutions of test sera and incubated 60 min at room temperature before addition to 10⁵ MT-4 human lymphoid cells in 96 well microtiter plates. The virus/cell mixtures were incubated for 7 days at 37°C and assayed for virus-mediated killing of cells by staining cultures with tetrazolium dye. Neutralization of virus is observed by prevention of virus-mediated cell death.

EXAMPLE 9

PROTECTION OF CHIMPANZEES UPON CHALLENGE WITH VIRULENT HIV-1:

The only animal HIV challenge model to date is with chimpanzees. While chimpanzees do not develop HIV-related immunodeficiency disease they can be infected with some HIV viral isolates. The most common strain used to date in this model is the IIIB strain (BH10) although challenge stocks for other isolates are being developed, e.g., for SF2. We envision vaccination of chimpanzees in an analogous manner to vaccination in other nonhuman primates using HIV *env* and *gag-pol* constructs derived from the HIV-1 IIIB strain (HXB2 clone) as described within this document to achieve anti-HIV humoral and cellular responses. While the BH10 challenge virus for chimpanzees is IIIB derived as are our vaccination construct genes, there is heterogeneity within this virus so that HXB2 is only one of at least three variations of IIIB present in the viral inoculum. Thus, the IIIB challenge experiment of HXB2 gene vaccinated monkeys is not completely homologous.

We are vaccinating chimpanzees 3-5 rounds with polynucleotide HIV gene vaccines with doses of 0.1-3 mg of plasmid/round. After characterization of vaccine-induced humoral and CTL anti-HIV responses these monkeys are challenged with 10 to 140 CID₅₀ (50% chimpanzee infectious dose) by an intravenous administration of HIV-1 IIIB inoculum diluted 1:25 in physiologic saline just prior to use. Infection of chimpanzees is monitored by detection of HIV-1 virus specific DNA sequences using DNA derived from PBMC

obtained from test chimpanzees. (see Example 10 for details). Vaccine-mediated protection can be described as a range of responses to challenge virus from complete sterilizing immunity (inability to detect virus post infection) to significant reductions and/or delay in viremia induced by the challenge stock. While sterilizing immunity is clearly the most preferred response to vaccination, reduced or delayed viremia may significantly influence onset of immunodeficiency disease in human vaccinees.

EXAMPLE 10

ISOLATION OF GENES FROM CLINICAL HIV ISOLATES:

HIV viral genes were cloned from infected PBMC's which had been activated by ConA treatment. The preferred method for obtaining the viral genes was by PCR amplification from infected cellular genome using specific oligomers flanking the desired genes. A second method for obtaining viral genes was by purification of viral RNA from the supernatants of infected cells and preparing cDNA from this material with subsequent PCR. This method was very analogous to that described above for cloning of the murine B7 gene except for the PCR oligomers used and random hexamers used to make cDNA rather than specific priming oligomers.

Genomic DNA was purified from infected cell pellets by lysis in STE solution (10 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8.0) to which Proteinase K and SDS were added to 0.1 mg/ml and 0.5% final concentrations, respectively. This mixture was incubated overnight at 56°C and extracted with 0.5 volumes of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was then precipitated by addition of sodium acetate to 0.3 M final concentration and two volumes of cold ethanol. After pelleting the DNA from solution the DNA was resuspended in 0.1X TE solution (1X TE = 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). At this point SDS was added to 0.1% with 2 U of RNase A with incubation for 30 minutes at 37°C. This solution was extracted with phenol/chloroform/isoamyl alcohol and then precipitated with ethanol as before. DNA was

suspended in 0.1 X TE and quantitated by measuring its ultraviolet absorbance at 260 nm. Samples were stored at -20°C until used for PCR.

5 PCR was performed using the Perkin-Elmer Cetus kit and procedure using the following sense and antisense oligomers for gp160: 5'-GA AAG AGC AGA AGA CAG TGG CAA TGA -3', SEQ.ID:52: and 5'-GGG CTT TGC TAA ATG GGT GGC AAG TGG CCC GGG C
10 ATG TGG-3', SEQ.ID:53:, respectively. These oligomers add an SrfI site at the 3'-terminus of the resulting DNA fragment. PCR-derived segments are cloned into either the V1Jns or V1R vaccination vectors and V3 regions as well as ligation junction sites confirmed by DNA sequencing.

15 EXAMPLE 11

SEQUENCES ACROSS VACCINE CONSTRUCT JUNCTIONS:

Genes were cloned according to Example 10. In each case, the junction sequences from the 5' promoter region (CMVintA) into the cloned gene was sequenced using the primer:
20 CMVinta primer 5'- CTA ACA GAC TGT TCC TTT CCA TG- 3', . SEQ. ID:54:, which generates the sequence of the coding sequence. This is contiguous with the terminator/coding sequence, the junction of which is also shown. This sequence was generated using the primer: BGH primer 5'- GGA GTG GCA CCT TCC AGG -3', SEQ. ID:55:.,
25 which generates the sequence of the non-coding strand. In every case, the sequence was checked against known sequences from GENBANK for cloned and sequenced genes from these or other HIV isolates. The position at which the junction occurs is demarcated by a "/", which does not represent any discontinuity in the sequence. The first "ATG"
30 encountered in each sequence is the translation initiation codon for the respective cloned gene. Each sequence provided represents a complete, available, expressible DNA construct for the designated HIV gene. The nomenclature follows the convention: "Vector name-HIV strain-gene". The biological efficacy of each of these constructs is shown in the same manner as in the foregoing Examples:

5 SEQUENCE ACROSS THE 5' JUNCTIONS OF CMV_{intA} AND THE
HIV GENES AND ACROSS THE 3' JUNCTIONS OF THE HIV
GENES AND THE BGH TERMINATOR EXPRESSION
CONSTRUCTS, USING DIFFERENT HIV STRAINS AND
PROTEINS:

1. V1Ins-revIIIIB:

10 SEQ.ID:56:

5'-GGA GAC AGC GACGAA GAC CTC CTC AAG GCA GTC AGA CTC ATC AAG-3'

rev....

(Sequence begins at the 5'- terminus within the PCR oligomer. See #7 below
for complete *rev* 5'- terminus sequence)

15 SEQ.ID:57:

5'-GAT GGC TGG CAA CTA GAA GGC ACA GCA GAT CT/GAT ATC GCA CTA

BGH

rev...

20 TTC TTT AGC TCC TGA CTC CAA TAT TGT-3'

2. V1Ins-gp160IIIIB:

SEQ.ID:58:

25 5'-CTT AGA TC/ A ACC ATG AGA GTG AAG GA GAA ATA TCA GCA CTT GTG

CMV_{inta}

gp160

GAG ATG GGG GTG GAG ATG GGG CAC CAT GCT CCT TGG GAT GTT GAT GAT
CTG TAG TGC TAC AGA AAA ATT GTG GGT-3'

30 SEQ.ID:59:

5'-CTG GCA ACT AGA AGG CAC AGC AGA TC/ A GAT AGT GTC CCC ATC TTA

BGH

gp160

TAG CAA AAT CCT TTC CAA GCC CTG TCT TAT TCT-3'

- 5 3. **pGEM-3-IRES**: [sequenced using SP6 (5'-GAT TTA GGT GAC ACT ATA G-3', SEQ.ID:60:) and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3', SEQ.ID:61:) primers, Promega Biotech]

SEQ.ID:62:

5'-CAT GCC TGC AGG TCG ACT CTA/ AAT TCC G...

10 **pGEM-3 (SP6) IRES**

SEQ.ID:63:

5'-A CCC GGG GAT CCT CT/ A GCG CGC TTG TCT CTT GTT CCA...

pGEM-3 (T7) IRES

- 15 4. **pGEM-3-IRES/revIIIB**: [sequenced using T7 sequencing primer (Promega) for *rev* 3'-end, and] IRES 3'- oligomer (5'-GG GAC GTG GTT TTC C-3', SEQ.ID:64:) for IRES/*rev* junction]

SEQ.ID:65:

20 5'-TAT GGC CAC AAC C/ AT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA

IRES *rev*

CCT CCT CAA GGC AGT CAG ACT -3'

SEQ.ID:66:

25 5'-CTC GAG CCA TGG GCC CCT/ AGA CTA TAG CGT GAT AAG AAA TCG AGG

pGEM-3 *rev*

ACT GAG GTT ATA ACA TCC TCT AAG GTG GTT ATA AAC TCC CGA AGG-3'

- 30 5. **pGEM-3-RRE/IRES/revIIIB**: [using SP6 sequencing oligomer (Promega) and IRES 5'- oligomer, 5'-G CTT CGG CCA GTA ACG-3', SEQ.ID:67:]

SEQ.ID:68:

5'-TTG CAT GCC TGC AGG T/ GGT ACA TGA TCA GAT ATC G CCC GGG / C

pGEM-3**RRE**

CGA GAT CTT CAG ACT TGG AGG AGG AGA TAT GAG GGA CAA TTG GAG-3'

5

IRES-5'

SEQ.ID:69:

5'-GGG GCG GAA TT/ T AGA GTC A/ ATT GAT CAG CTT GTG TAA TTG TTA

RRE-3'

10

ATT TCT CTG TCC CAC TCC ATC CAG GTC GTG TGA TTC...-3'

6. **V1Ins-(tat/rev SD)**: [used for V1Ins-gp160^{IIIB}/IRES/rev^{IIIB} (SD) and V1Ins-gp160^{IIIB}(SD); sequenced using an oligomer complementary to gp160 reading towards 5'-end of gp160 and into CMVintA: 5-CCA TCT CCA CAA GTG CTG-3', SEQ.ID:70:]

15

SEQ.ID:71:

5'-AGA TCT A AGG ACG GTG ACT GCA / TGT ACT ACT TAC TGC TTT GAT

20

CMVintA***tat/rev SD***

AGA GGA CGG TGA / CTG CAG AAA AGA CCC ATG GAA A-3'

CMVintA

25

7. **V1Ins-gp160^{IIIB}/IRES/rev^{IIIB} (SD)**: [gp160/IRES junction sequenced using IRES 5'- oligomer, 5'-G CTT CGG CCA GTA ACG-3', SEQ.ID:72:]

SEQ.ID:73:

30

5'-GGC ACA GCA GAT C/ AG ATG GGG ATC TGA TA TCG CAC TAT TCT TTA

BGH***rev***

GCT CCT GAC TCC TGA CTC-3'

SEQ.ID:74:

5'-GGA ATT/ TGA GTC ATC / CCC ATC TTA TAG CAA AAT CCT TTC CAA -3'
IRES **gp160**

8. V1Ins-gag-prtIIIB (SD):

5

SEQ.ID:75:

5'-CTT AGA TC/ C CCG CAC GGC AAG AGG CGA GGG GCG GCG ACT GGT-3'
CMVintA **gag (SD)**

10

SEQ.ID:76:

5'-GGC ACA GCA GAT C/ CGC CCG GGC TTA CAT CTC TGT ACA AAT TTC TAC
BGH **prt**

TAA TGC TTT TAT TTT TCT TCT GTC...-3'

15

9. V1Ins-gag-prtIIIB:

SEQ.ID:77:

20

5'-CTT AGA TC/ CAC CAT GGG TGC GAG AGC GTC AGT ATT AA GCG GGG
CMVintA **gag**

GGA GAA TTA GAT CGA TGG GAA AAA ATT...-3'

SEQ.ID:78:

25

5'-GGC ACA GCA GAT C/ CGC CCG GGC TTA CAT CTC TGT ACA AAT TTC TAC
BGH **prt**

TAA TGC TTT TAT TTT TCT TCT GTC...-3'

30

10. V1Ins-tPA:

SEQ.ID:79:

5'-TCA CCG TCC TTA GAT C/ ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC
CMVintA **tPA leader**

TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC GA/ G ATC

BGH

5

TGC TGT GCC TTC TAG TTG CCA GCC-3'

11. V1Jns-tPA-gp120MN:

10

SEQ.ID:80:

5'-TTC GTT TCG CCC AGC GA/ TCA CAG AAA AAT TGT GGG TCA CAG TC-3'

tPA

gp120MN

SEQ.ID:81:

15

5'-GGC ACA GCA GAT C/ CAC GTG TTA GCG CTT TTC TCT CTC CAC CAC-3'

BGH

gp120MN

12. V1J-SIVMAC251 p28 gag

20

SEQ.ID:82:

5'-TCA CCG TCC TTA GAT CT/ ACC ATG GGA CCA GTA CAA CAA ATA GGT

CMVintA

p28 gag...

GGT AAC TAT GTC CAC CTG CCA TTA AGC CCG AGA ACA-3'

25

SEQ.ID:83:

5'-GGC ACA GCA GAT CT/ TTA CAT TAA TCT AGC CTT CTG TCC CGG TCC-3'

BGH

p28 gag

30

13. V1J-SIVMAC251nef

SEQ.ID:84:

5'-TCA CCG TCC TTA GAT C/ GGT ACA ACC ATG GGT GGA GCT ATT TCC ATG

CMVintA

nef.....

AGG CAA TCC AAG CCG GCT GGA GAT CTG ACA GAA A-3'

SEQ.ID:85:

5 5'-GGC ACA GCA GAT CA/ C CTA GGT TAG CCT TCT TCT AAC CTC TTC CTC
BGH *n ef....*
 TGA CAG GCC TGA CTT GCT TCC AAC TCT TCT GGG TAT CTA G-3'

14. V1Ins-tat/rev/env:

10 SEQ.ID:86:
 5'-ACC GTC CTT AGA T/ TC GAC ATA GCA GAA TAG GCG TTA CTC GAC AGA
CMVintA *tat/rev/env*

15 GGA GAG CAA GAA ATG GAG CCA GTA GAT CCT AGA CTA GAG CCC TGG-3'

SEQ.ID:87:

20 5'-GGC ACA GCA GAT C/ C GAG ATG CTG CTC CCA CCC CAT CTG CTG-3'
BGH *tat/rev/env*

EXAMPLE 12

T CELL PROLIFERATION ASSAYS:

PBMCs can be obtained as described in Example 6 from above and tested for recall responses to specific antigen as determined by proliferation within the PBMC population. Proliferation is
 25 monitored using ³H-thymidine which is added to the cell cultures for the last 18-24 hours of incubation before harvest. Cell harvesters retain isotope-containing DNA on filters if proliferation has occurred while quiescent cells do not incorporate the isotope which is not retained on
 30 the filter in free form. For either rodent or primate species 4 X 10⁵ cells are plated in 96 well microtiter plates in a total of 200 µl of complete media (RPMI/10% fetal calf serum). Background proliferation responses are determined using PBMCs and media alone while nonspecific responses are generated by using lectins such as phytohaemagglutinin (PHA) or concanavalin A (ConA) at 1- 5 µg/ml concentrations to serve as a positive control. Specific antigen consists of

either known peptide epitopes, purified protein, or inactivated virus. Antigen concentrations range from 1- 10 μ M for peptides and 1-10 μ g/ml for protein. Lectin-induced proliferation peaks at 3-5 days of cell culture incubation while antigen-specific responses peak at 5-7 days. Specific proliferation occurs when radiation counts are obtained which are at least three-fold over the media background and is often given as a ratio to background, or Stimulation Index (SI). HIV gp160 is known to contain several peptides known to cause T cell proliferation of gp160/gp120 immunized or HIV-infected individuals. The most commonly used of these are: T1 (LysGlnIleIleAsnMetTrpGlnGluValGlyLysAlaMetTyrAla, SEQ.ID:88:); T2 (HisGluAspIleIleSerLeuTrpAspGlnSerLeuLys, SEQ.ID:89:); and, TH4 (AspArgValIleGluValValGlnGlyAalTyrArgAlaIleArg, SEQ.ID:90:). These peptides have been demonstrated to stimulate proliferation of PBMC from antigen-sensitized mice, nonhuman primates, and humans.

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EXAMPLE 13

Vector V1R Preparation

In an effort to continue to optimize our basic vaccination vector, we prepared a derivative of V1Jns which was designated as V1R. The purpose for this vector construction was to obtain a minimum-sized vaccine vector, i.e., without unnecessary DNA sequences, which still retained the overall optimized heterologous gene expression characteristics and high plasmid yields that V1J and V1Jns

afford. We determined from the literature as well as by experiment that (1) regions within the pUC backbone comprising the *E. coli* origin of replication could be removed without affecting plasmid yield from bacteria; (2) the 3'-region of the *kan^r* gene following the kanamycin open reading frame could be removed if a bacterial terminator was inserted in its stead; and, (3) ~300 bp from the 3'- half of the BGH terminator could be removed without affecting its regulatory function (following the original KpnI restriction enzyme site within the BGH element).

V1R was constructed by using PCR to synthesize three segments of DNA from V1Jns representing the CMVintA promoter/BGH terminator, origin of replication, and kanamycin resistance elements, respectively. Restriction enzymes unique for each segment were added to each segment end using the PCR oligomers: SspI and XhoI for CMVintA/BGH; EcoRV and BamHI for the *kan^r* gene; and, BclI and Sall for the *ori^r*. These enzyme sites were chosen because they allow directional ligation of each of the PCR-derived DNA segments with subsequent loss of each site: EcoRV and SspI leave blunt-ended DNAs which are compatible for ligation while BamHI and BclI leave complementary overhangs as do Sall and XhoI. After obtaining these segments by PCR each segment was digested with the appropriate restriction enzymes indicated above and then ligated together in a single reaction mixture containing all three DNA segments. The 5'-end of the *ori^r* was designed to include the T2 rho independent terminator sequence that is normally found in this region so that it could provide termination information for the kanamycin resistance gene. The ligated product was confirmed by restriction enzyme digestion (>8 enzymes) as well as by DNA sequencing of the ligation junctions. DNA plasmid yields and heterologous expression using viral genes within V1R appear similar to V1Jns. The net reduction in vector size achieved was 1346 bp (V1Jns = 4.86 kb; V1R = 3.52 kb), see figure 11, SEQ.ID:45:.

PCR oligomer sequences used to synthesize V1R (restriction enzyme sites are underlined and identified in brackets following sequence):

- (1) 5'-GGT ACA AAT ATT GG CTA TTG GCC ATT GCA TAC G-3' [SspI],
SEQ.ID:91;
- 5 (2) 5'-CCA CAT CTC GAG GAA CCG GGT CAA TTC TTC AGC ACC-3' [XhoI],
SEQ.ID:92:
(for CMVintA/BGH segment)
- (3) 5'-GGT ACA GAT ATC GGA AAG CCA CGT TGT GTC TCA AAA TC-
10 3'[EcoRV], SEQ.ID:93:
(4) 5'-CCA CAT GGA TCC G TAA TGC TCT GCC AGT GTT ACA ACC-3'
[BamHI], SEQ.ID:94:
(for kanamycin resistance gene segment)
- (5) 5'-GGT ACA TGA TCA CGT AGA AAA GAT CAA AGG ATC TTC TTG-
15 3'[BclI], SEQ.ID:95;
(6) 5'-CCA CAT GTC GAC CC GTA AAA AGG CCG CGT TGC TGG-3' [SalI],
SEQ.ID:96:
(for E. coli origin of replication)
- 20 Ligation junctions were sequenced for V1R using the following
oligomers:
5'-GAG CCA ATA TAA ATG TAC-3', SEQ.ID:97: [CMVintA/kan^r
junction]
5'-CAA TAG CAG GCA TGC-3', SEQ.ID:98: [BGH/ori junction]
25 5'-G CAA GCA GCA GAT TAC-3', SEQ.ID:99: [ori/kan^r junction]

EXAMPLE 14

- The HIV genes which appear to be the most important for
PNV development are env and gag. Both env and gag require the HIV
30 regulatory protein, rev, for either viral or heterologous expression.
Because efficient expression of these gene products is essential for PNV
function, two types of vectors, rev-dependent and rev-independent,
were tested for vaccination purposes. Unless stated otherwise, all genes
were derived from the HIV-1 (IIIB) laboratory isolate.

A. env: Depending upon how large a gene segment is used, varying efficiencies of rev-independent envy expression may be achieved by replacing the native leader peptide of env with the leader peptide from the tissue-specific plasminogen activator (tPA) gene and expressing the resulting chimeric gene behind the CMV promoter with the CMV intronA. V1Jns-tPA-gp120 is an example of a secreted gp120 vector constructed in this fashion which functions to yield anti-gp120 immune responses in vaccinated mice and monkeys.

Published reports indicate that membrane-anchored proteins may induce a more substantial antibody responses compared to secreted proteins. Membrane-anchored proteins may also induce antibody responses to additional immune epitopes. To test this hypothesis, V1Jns-tPA-gp160 and V1Jns-rev/env were prepared. The tPA-gp160 vector produced detectable quantities of gp160 and gp120, without the addition of rev, as shown by immunoblot analysis of RD cells transfected in vitro, although expression was much lower than that obtained for rev/env, a rev-dependent gp160-expressing plasmid. This may be due to the presence of inhibitory regions, which confer rev dependence upon the gp160 transcript occur at multiple sites within gp160 including at the COOH-terminus of gp41.

Vectors containing truncated forms of tPA-gp160, tPA-gp143 and tPA-gp150, designed to increase the overall expression of env by elimination of these inhibitory sequences, were prepared. The truncated gp160 vectors lack intracellular gp41 regions containing peptide motifs (such as leu-leu) which are known to cause diversion of membrane proteins to the lysosomes rather than the cell surface. Thus, gp143 and gp150 may be expected to increase the transport of protein to the cell surface compared to full-length gp160 where these proteins may be better able to elicit anti-gp160 antibodies following DNA vaccination.

A quantitative ELISA for gp160/gp120 expression in cell transfectants was developed to determine the relative expression capabilities for these vectors as well as for an additional vector which combines the features of tPA-gp160 and rev/env (vector rev/tPA-

gp160). In vitro transfection of 293 cells followed by quantitation of cell-associated vs. secreted/released gp120 yielded the following results:

(1) for the analogous plasmid pair, rev/env and rev/tPA-gp160, substitution of the native leader peptide in gp160 with the tPA leader sequence did not increase the total expression of gp160 or the amount of released gp120. This suggests that the leader peptide is not responsible for inefficient trafficking of gp160 to the cell surface in these cells.

(2) tPA-gp160 expresses 5-10X less gp160 than rev/env with similar proportions retained intracellularly vs. trafficked to the cell surface.

(3) tPA-gp143 gave 3-6X greater secretion of gp120 than rev/env with only low levels of cell-associated gp143 confirming that the cytoplasmic tail of gp160 causes intracellular retention of gp160 which can be overcome by partial deletion of this sequence.

(4) tPA-gp150 gave only low levels of gp160 in both cells and media, indicating either a problem with this construct or inherent instability of the truncated protein.

tPA-gp120 derived from a primary HIV isolate (containing the North American consensus V3 peptide loop; macrophage tropic and nonsyncytia-inducing phenotypes) gave high expression/secretion of gp120 with transfected 293 cells demonstrating that it was cloned in a functional form.

EXAMPLE 15

Serological Assays

A. Antibody Responses:

1. gp120 PNVs

An ID vs. IM vaccination experiment in mice was completed using V1Jns-tPA-gp120 (100, 10, 1 µg: 3X). ID vaccination appeared superior at the lower doses following initial rounds but all doses were equivalent after three rounds.

Rhesus monkeys (RHM) and African green monkeys (AGM) were vaccinated with the V1Jns-tPA-gp120 (MN) PNV. Peak

GMTs for gp120 antibodies differed by more than five-fold between these two primate species: 1780 (AGM) and 310 (RHM). These results indicate that substantially larger antibody titers can be elicited in AGM compared to RHM and suggest that higher HIV neutralization titers may be obtained by AGM vaccination.

2. gp160 PNVs: V1Jns-rev/env vaccination (IM) of mice did not yield antibodies to gp160 until three injections while ID vaccination yielded responses after one round which remained higher than those produced by IM throughout the experiment (GMTs = 2115 (ID) and 95 (IM); 200 µg/mouse). This suggests that rev-dependent constructs can function as immunogens better by the ID route.

RHM receiving ID or IM inoculations with V1Jns-rev/env showed peak GMTs = 790 and 140, respectively, following 4-5 inoculations (2 mg/round). These results agree with those found for mice showing that this rev dependent PNV has greater efficacy for antibody generation by ID vaccination although the rev-independent construct V1Jns-tPA-gp120 did not. RHM receiving tPA-gp160 DNA (IM) showed lower, more variable antibody responses than those receiving rev/env which corroborate our determination that this vector expresses gp160 4-7X less efficiently than rev/env.

B. In Vitro Virus Neutralization

An infectivity reduction neutralization assay (p24 gag production readout) using HIV(MN) as a virus source was performed by Quality Biologicals, Inc. (QBI). At low virus input (100 TCID₅₀) complete neutralization was seen at 1/10 dilutions of sera for all three antisera with at least 80-90 % reduction in virus production observed in all samples up to 1/80 dilutions as compared to matched prebleed sera. However, at higher virus input (1000 TCID₅₀), no neutralization was observed for any sample.

RHM were tested for HIV (IIIB) neutralization (QBI), using 100 TCID₅₀ of input virus, following vaccinations with tPA-gp120 (IIIB) DNA. In two different experiments the best neutralization results were obtained at serum dilutions of 10 (40-99% reduction of p24 gag) with gag reduction observed in some samples at dilutions as high

as 80-fold. The most consistent samples in this assay had anti-gp120 antibody ELISA endpoint titers of at least 2000-3000.

RHM were similarly tested for HIV (IIIB) neutralization (QBI) following vaccinations with rev/env DNA. Overall, low levels of neutralization were observed: two of three RHM showed neutralization ranging up to 84% at a serum dilution of 10 with p24 gag reduction observed at subsequent dilutions of 20 or 40 while one sample did not show any evidence of neutralization. These samples had anti-gp120 antibody ELISA titers of 700-800 indicating that this is the minimum useful titer range for testing sera derived from gp160 DNA vaccine experiments in neutralization assays.

C. Facilitators for Enhanced Immunity

Several experiments were initiated to test plasmid DNA formulations which have been reported to enhance DNA uptake following vaccination and increase either reporter gene expression or immune responses in mouse or monkey vaccinees. Hypertonic sucrose (up to 20-25%, w/v) DNA solutions have been reported to give more uniform distribution of DNA uptake, as evidenced by reporter gene expression, and was used in experiments in which substantial gp160-specific antibodies were elicited in rodents and nonhuman primates vaccinated with a rev/gp160 plasmid. The anesthetic, bupivacaine (0.25-0.75%, w/v), has also been reported to significantly enhance DNA vaccine-mediated immune responses in mice and nonhuman primates when used either as a pretreatment for IM injection, or as by co-injection with DNA in isotonic saline solution.

Our initial results with bupivacaine showed that substantial mortality was caused by IM treatment with 0.5% solutions. Mortality varied depending on the volume of solution used and whether the mice were injected while under anesthetic (≥ 0.1 mL w/o anesthetic gave highest mortality). Our experiments have used 0.25% solutions without significant mortality either as a pre-treatment or a co-treatment and using gp120 or rev/env PNVs. A preliminary experiment using bupivacaine as a pre-treatment for three vaccination rounds did not show any enhancement of immune responses relative to control mice

while a larger experiment using both ID and IM sites as a pre-treatment or co-treatment has not shown any increased antibody levels following one injection and appeared to decrease antibody responses in some groups. Three vaccinations are planned in the current study.

This sucrose formulation experiment tested a variety of conditions described in the literature. Sucrose concentration was tested at 10, 15, 20, and 25% in saline or PBS solution containing 0.1 mg/mL of tPA-gp120 plasmid. All samples were tested as a co-injection by IM or ID routes except for a 25% sucrose/PBS group that received this solution 15-30 minutes prior to IM DNA/PBS injection. Serum data derived from bleeds following the first vaccination did not show any enhancement of antibody responses.

EXAMPLE 16

T Lymphocyte Responses:

A. Proliferation and Cytokine Secretion

T lymphocytes which have been primed in vivo with antigen can proliferate and secrete cytokines during in vitro cell culture after exogenous addition of priming antigen. Responding T cells usually have a MHC Class II-restricted, CD4+ (helper) phenotype. Helper T cells can be functionally grouped according to the types of cytokines they secrete following stimulation by antigen: TH1 cells secrete primarily IL-2 and g-interferon while TH2 cells are associated with IL-4, IL-5, and IL-10 secretion. TH1 lymphocytes and cytokines promote cellular immunity, including CTL and DTH responses, while TH2 cells and cytokines promote B cell activation for humoral immunity. We have previously tested for these responses in mice and nonhuman primates (AGM and RHM), using rgp120III_B for antigen in vitro, after vaccination with HIV tPA-gp120 PNVs and shown that T cells from vaccinees of both species exhibit proliferative responses to gp120 in vitro and that these responses are TH1-like and long-lived (> 6 months) in mice. These studies were continued with a rev PNV.

1. mouse studies: Mice vaccinated either 3X or 1X with 200 µg V1Jns-rev were tested for in vitro proliferation to recombinant

rev (r-rev) protein. Mice vaccinated 3X showed stimulation indices (SI: ratio of proliferation of immune cells with and without immunizing antigen) of 9-12 while mice receiving 1X were the same as background (SIs = 2-3). Splenic T cells from all rev vaccinees, but not control mice, secreted g-interferon in response to r-rev antigen (2.4-2.8 ng/ml, 3X; 0.4-0.7 ng/ml, 1X) while no IL-4 was detected in culture supernatants (detection sensitivities = 47 pg/ml and 15 pg/ml for g-interferon and IL-4, respectively) showing these T cell responses to be TH1-like in nature as we found for gp120 DNA vaccinees. Cytokine secretion may be a more sensitive assay than proliferation to specific antigen for determining T cell memory responses. Similar results were found for mice tested at least six months post vaccination. Antibodies to rev were not detected in any vaccinee sera as may be expected for this intracellular protein.

2. Monkey Studies: Three RHM showed strong in vitro T cell proliferation (SIs = 9-30) to r-rev following two vaccinations with V1Jns-rev. No anti-rev antibodies were detected in any monkeys. These results corroborate the above mouse/rev experiments and confirm that strong T cell responses can be induced by rev PNVs without concomitant induction of antibody responses.

Further experiments using tPA-gp120 DNA vaccination of RHM showed that (i) in vitro T cell proliferation to rgp120 was obtained following one vaccination; (ii) primary responses were boosted following a second vaccination; and, (iii) similar proliferations were obtained with these vaccinees as for SHIV-infected RHM (SIs = 5-70 and 5-35, respectively).

B. Anti-env Cytotoxic T Lymphocytes

Two of four RHM monkeys vaccinated with tPA-gp120 (IM) and gp160/IRES/rev (ID) PNVs showed significant CTL activities (> 20% lysis at 10:1 E/T) against homologous target cells six weeks following one vaccination. Two weeks post a second vaccination all four monkeys showed cytotoxicities ranging from 20 -35% lysis at 20:1 E/T. All CTL activities in this assay design were MHC Class I

restricted: removal of CD8+ T cells completely removed cytotoxicities
in all four monkeys. CTL responses waned over several months and
were boosted to \geq original levels with subsequent re-vaccination. These
CTL activities were characterized as the most potent for vaccine-
mediated responses observed in RHM.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Shiver, John W
Liu, Margaret A
Perry, Helen C
- (ii) TITLE OF INVENTION: COORDINATE IN VIVO GENE EXPRESSION
- (iii) NUMBER OF SEQUENCES: 100
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 - (E) COUNTRY: United States of America
 - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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 - (C) REFERENCE/DOCKET NUMBER: 19188Y
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- 87 -

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys	Thr	Arg	Pro	Asn	Asn	Asn	Thr	Arg	Lys	Ser	Ile	His	Ile	Gly	Pro
1				5					10					15	
Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Gly	Glu	Ile	Ile	Gly	Asp	Ile	Arg	Gln
			20					25					30		
Ala	His	Cys													
		35													

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys	Thr	Arg	Pro	Asn	Tyr	Asn	Lys	Arg	Lys	Arg	Ile	His	Ile	Gly	Pro
1				5					10					15	
Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Lys	Asn	Ile	Ile	Gly	Thr	Ile	Arg	Gln
			20					25					30		
Ala	His	Cys													
		35													

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

- 88 -

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys	Thr	Arg	Pro	Asn	Asn	Asn	Thr	Arg	Lys	Arg	Ile	Arg	Ile	Gln	Arg
1				5					10					15	
Gly	Pro	Gly	Arg	Ala	Phe	Val	Thr	Ile	Gly	Lys	Ile	Gly	Asn	Met	Arg
			20					25					30		
Gln	Ala	His	Cys												
			35												

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys	Thr	Arg	Pro	Asn	Asn	Asn	Thr	Arg	Lys	Gly	Ile	His	Ile	Gly	Pro
1				5					10					15	
Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Gly	Lys	Ile	Ile	Gly	Asn	Ile	Arg	Gln
			20					25					30		
Ala	His	Cys													
		35													

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

- 89 -

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys	Thr	Arg	Pro	Ser	Asn	Asn	Asn	Thr	Arg	Lys	Ser	Ile	His	Ile	Gly
1				5				10					15		
Pro	Gly	Lys	Ala	Phe	Tyr	Ala	Thr	Gly	Ala	Ile	Ile	Gly	Asp	Ile	Arg
			20					25					30		
Gln	Ala	His	Cys												
			35												

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: both

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys	Thr	Arg	Pro	Asn	Asn	Asn	Thr	Arg	Arg	Ser	Ile	His	Ile	Ala	Pro
1				5				10					15		
Gly	Arg	Ala	Phe	Tyr	Ala	Thr	Gly	Asp	Ile	Ile	Gly	Asp	Ile	Arg	Gln
			20					25					30		
Ala	His	Cys													
			35												

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

- 90 -

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTATATAAGC AGAGCTCGTT TAG

23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTAAGCAAAGA TCTAAGGACG GTGACTGCAG

30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTATGTGTCTT GAAAATGAGC GTGGAGATTG GGCTCGCAC

39

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

- 91 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGCGAGCCC AATCTCCACG CTCATTTTCA GACACATAC

39

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGTACAAGAT CTACTATAGG GAGACCGGAA TTCCGC

36

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4432 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA

60

CAGCTTGTCT	GTAAGCGGAT	GCCGGGAGCA	GACAAGCCCG	TCAGGGCGCG	TCAGCGGGTG	120
TTGGCGGGTG	TCGGGGCTGG	CTTAACATATG	CGGCATCAGA	GCAGATTGTA	CTGAGAGTGC	180
ACCATATGCG	GTGTGAAATA	CCGCACAGAT	GCGTAAGGAG	AAAATACCGC	ATCAGATTGG	240
CTATTGGCCA	TTGCATACGT	TGTATCCATA	TCATAATATG	TACATTTATA	TTGGCTCATG	300
TCCAACATTA	CCGCCATGTT	GACATTGATT	ATTGACTAGT	TATTAATAGT	AATCAATTAC	360
GGGGTCATTA	GTTTCATAGCC	CATATATGGA	GTTCCGCGTT	ACATAACTTA	CGGTAAATGG	420
CCCGCCTGGC	TGACCGCCCA	ACGACCCCGC	CCCATTGACG	TCAATAATGA	CGTATGTTCC	480
CATAGTAACG	CCAATAGGGA	CTTTCCATTG	ACGTCAATGG	GTGGAGTATT	TACGGTAAAC	540
TGCCCCACTTG	GCAGTACATC	AAGTGTATCA	TATGCCAAGT	ACGCCCCCTA	TTGACGTCAA	600
TGACGGTAAA	TGGCCCGCCT	GGCATTATGC	CCAGTACATG	ACCTTATGGG	ACTTTCCTAC	660
TTGGCAGTAC	ATCTACGTAT	TAGTCATCGC	TATTACCATG	GTGATGCGGT	TTTGGCAGTA	720
CATCAATGGG	CGTGGATAGC	GGTTTGA CTC	ACGGGGATTT	CCAAGTCTCC	ACCCCATTGA	780
CGTCAATGGG	AGTTTGT TTT	GGCACCAAAA	TCAACGGGAC	TTTCCAAAAAT	GTCGTAACAA	840
CTCCGCCCCA	TTGACGCAAA	TGGGCGGTAG	GCGTGTACGG	TGGGAGGTCT	ATATAAGCAG	900
AGCTCGTTTA	GTGAACCGTC	AGATCGCCTG	GAGACGCCAT	CCACGCTGTT	TTGACCTCCA	960
TAGAAGACAC	CGGGACCGAT	CCAGCCTCCG	CGGCCGGGAA	CGGTGCATTG	GAACGCGGAT	1020
TCCCCGTGCC	AAGAGTGACG	TAAGTACCGC	CTATAGAGTC	TATAGGCCCA	CCCCCTTGGC	1080
TTCTTATGCA	TGCTATACTG	TTTTTGGCTT	GGGGTCTATA	CACCCCCGCT	TCCTCATGTT	1140
ATAGGTGATG	GTATAGCTTA	GCCTATAGGT	GTGGGTATT	GACCATTTATT	GACCACTCCC	1200
CTATTGGTGA	CGATACTTTC	CATTACTAAT	CCATAACATG	GCTCTTTGCC	ACAACTCTCT	1260
TTATTGGCTA	TATGCCAATA	CACTGTCCTT	CAGAGACTGA	CACGGACTCT	GTATTTTTTAC	1320
AGGATGGGGT	CTCATTTTATT	ATTTACAAAT	TCACATATAC	AACACCACCG	TCCCCAGTGC	1380
CCGCAGTTTT	TATTAAACAT	AACGTGGGAT	CTCCACGCGA	ATCTCGGGTA	CGTGTTCGGG	1440
ACATGGGCTC	TTCTCCGGTA	GCGGCGGAGC	TTCTACATCC	GAGCCCTGCT	CCCATGCCTC	1500
CAGCGACTCA	TGGTCGCTCG	GCAGCTCCTT	GCTCCTAACA	GTGGAGGCCA	GACTTAGGCA	1560
CAGCACGATG	CCCACCACCA	CCAGTGTGCC	GCACAAGGCC	GTGGCGGTAG	GGTATGTGTC	1620
TGAAAATGAG	CTCGGGGAGC	GGGCTTGAC	CGCTGACGCA	TTTGGAAGAC	TTAAGGCAGC	1680
GGCAGAAGAA	GATGCAGGCA	GCTGAGTTGT	TGTGTTCTGA	TAAGAGTCAG	AGGTA ACTCC	1740

CGTTGCGGTG	CTGTAAACGG	TGGAGGGCAG	TGTAGTCTGA	GCAGTACTCG	TTGCTGCCGC	1800
GCGCGCCACC	AGACATAATA	GCTGACAGAC	TAACAGACTG	TTCTTTTCCA	TGGGTCTTTT	1860
CTGCAGTCAC	CGTCCTTAGA	TCTGCTGTGC	CTTCTAGTTG	CCAGCCATCT	GTTGTTTGCC	1920
CCTCCCCCGT	GCCTTCCTTG	ACCCTGGAAG	GTGCCACTCC	CACTGTCCTT	TCCTAATAAA	1980
ATGAGGAAAT	TGCATCGCAT	TGTCTGAGTA	GGTGTCAATC	TATTCTGGGG	GGTGGGGTGG	2040
GGCAGCACAG	CAAGGGGGAG	GATTGGGAAG	ACAATAGCAG	GCATGCTGGG	GATGCGGTGG	2100
GCTCTATGGG	TACCCAGGTG	CTGAAGAATT	GACCCGGTTC	CTCTGGGCC	AGAAAGAAGC	2160
AGGCACATCC	CCTTCTCTGT	GACACACCCT	GTCCACGCCC	CTGGTTCTTA	GTTCCAGCCC	2220
CACTCATAGG	AACTCATAG	CTCAGGAGGG	CTCCGCCTTC	AATCCCACCC	GCTAAAGTAC	2280
TTGGAGCGGT	CTCTCCCTCC	CTCATCAGCC	CACCAAACCA	AACCTAGCCT	CCAAGAGTGG	2340
GAAGAAATTA	AAGCAAGATA	GGCTATTAAG	TGCAGAGGGA	GAGAAAATGC	CTCCAACATG	2400
TGAGGAAGTA	ATGAGAGAAA	TCATAGAATT	TCTTCCGCTT	CCTCGCTCAC	TGACTCGCTG	2460
CGCTCGGTCTG	TTCGGCTGCG	GCGAGCGGTA	TCAGCTCACT	CAAAGGCGGT	AATACGGTTA	2520
TCCACAGAAT	CAGGGGATAA	CGCAGGAAAG	AACATGTGAG	CAAAGGCCA	GCAAAAGGCC	2580
AGGAACCGTA	AAAAGGCCGC	GTTGCTGGCG	TTTTTCCATA	GGCTCCGCCC	CCCTGACGAG	2640
CATCAGAAAA	ATCGACGCTC	AAGTCAGAGG	TGGCGAAACC	CGACAGGACT	ATAAAGATAC	2700
CAGGCGTTTC	CCCCTGGAAG	CTCCCTCGTG	CGCTCTCCTG	TTCCGACCCT	GCCGCTTACC	2760
GGATACCTGT	CCGCCTTTCT	CCCTTCGGGA	AGCGTGGCGC	TTTCTCAATG	CTCAGCTGT	2820
AGGTATCTCA	GTTGCGGTGA	GGTCGTTGCG	TCCAAGCTGG	GCTGTGTGCA	CGAACCCCCC	2880
GTTCAGCCCG	ACCGCTGCGC	CTTATCCGGT	AACTATCGTC	TTGAGTCCAA	CCCGGTAAGA	2940
CACGACTTAT	CGCCACTGGC	AGCAGCCACT	GGTAACAGGA	TTAGCAGAGC	GAGGTATGTA	3000
GGCGGTGCTA	CAGAGTTCTT	GAAGTGGTGG	CCTAACTACG	GCTACACTAG	AAGGACAGTA	3060
TTTGGTATCT	GCGCTCTGCT	GAAGCCAGTT	ACCTTCGGAA	AAAGAGTTGG	TAGCTCTTGA	3120
TCCGGCAAAC	AAACCACCGC	TGGTAGCGGT	GGTTTTTTTG	TTTGCAAGCA	GCAGATTACG	3180
CGCAGAAAAA	AAGGATCTCA	AGAAGATCCT	TTGATCTTTT	CTACGGGGTC	TGACGCTCAG	3240
TGGAACGAAA	ACTCACGTTA	AGGGATTTTG	GTCATGAGAT	TATCAAAAAG	GATCTTCACC	3300
TAGATCCTTT	TAAATTAAAA	ATGAAGTTTT	AAATCAATCT	AAAGTATATA	TGAGTAAACT	3360
TGGTCTGACA	GTTACCAATG	CTTAATCAGT	GAGGCACCTA	TCTCAGCGAT	CTGTCTATTT	3420

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CGTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA	3480
CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA	3540
TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC	3600
GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT	3660
AGTTTGCGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT	3720
ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG	3780
TGCAAAAAAG CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA	3840
GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA	3900
AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG	3960
CGACCGAGTT GCTCTTGCCC GGCCTCAATA CGGGATAATA CCGCGCCACA TAGCAGAACT	4020
TTAAAAGTGC TCATCATTTG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG	4080
CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT	4140
ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA	4200
ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC TTTTCAATA TTATTGAAGC	4260
ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA	4320
CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT	4380
ATTATCATGA CATTAACTA TAAAAATAGG CGTATCACGA GGCCCTTTTCG TC	4432

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2196 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTGGCTATT GGCCATTGCA TACGTTGTAT CCATATCATA ATATGTACAT TTATATTGGC	60
TCATGTCCAA CATTACCGCC ATGTTGACAT TGATTATTGA CTAGTTATTA ATAGTAATCA	120

ATTACGGGGT CATTAGTTCA TAGCCCATAT ATGGAGTTCC GCGTTACATA ACTTACGGTA	180
AATGGCCCCG CTGGCTGACC GCCCAACGAC CCCC GCCCAT TGACGTCAAT AATGACGTAT	240
GTTCCCATAG TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA GTATTTACGG	300
TAAACTGCCC ACTTGGCAGT ACATCAAGTG TATCATATGC CAAGTACGCC CCCTATTGAC	360
GTCAATGACG GTAAATGGCC CGCCTGGCAT TATGCCCAGT ACATGACCTT ATGGGACTTT	420
CCTACTTGGC AGTACATCTA CGTATTAGTC ATCGCTATTA CCATGGTGAT GCGGTTTTGG	480
CAGTACATCA ATGGGCGTGG ATAGCGGTTT GACTCACGGG GATTTCCAAG TCTCCACCCC	540
ATTGACGTCA ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC AAAATGTCGT	600
AACAAC TCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA GGTCTATATA	660
AGCAGAGCTC GTTTAGTGAA CCGTCAGATC GCCTGGAGAC GCCATCCACG CTGTTTTGAC	720
CTCCATAGAA GACACCGGGA CCGATCCAGC CTCCGCGGCC GGGAACGGTG CATTGGAACG	780
CGGATTCCCC GTGCCAAGAG TGACGTAAGT ACCGCCTATA GAGTCTATAG GCCCACCCCC	840
TTGGCTTCTT ATGCATGCTA TACTGTTTTT GGCTTGGGGT CTATACACCC CCGCTTCCTC	900
ATGTTATAGG TGATGGTATA GCTTAGCCTA TAGGTGTGGG TTATTGACCA TTATTGACCA	960
CTCCCC TATT GGTGACGATA CTMTCCATTA CTAATCCATA ACATGGCTCT TTGCCACAAC	1020
TCTCTTTATT GGCTATATGC CAATACACTG TCCTTCAGAG ACTGACACGG ACTCTGTATT	1080
TTTACAGGAT GGGGTCTCAT TTATTATTTA CAAATTCACA TATACAACAC CACCGTCCCC	1140
AGTGCCCGCA GTTTTTATTA AACATAACGT GGGATCTCCA CGCGAATCTC GGGTACGTGT	1200
TCCGGACATG GGCTCTTCTC CGGTAGCGGC GGAGCTTCTA CATCCGAGCC CTGCTCCCAT	1260
GCCTCCAGCG ACTCATGGTC GCTCGGCAGC TCCTTGCTCC TAACAGTGGA GGCCAGACTT	1320
AGGCACAGCA CGATGCCCCAC CACCACCAGT GTGCCGCACA AGGCCGTGGC GGTAGGGTAT	1380
GTGTCTGAAA ATGAGCTCGG GGAGCGGGCT TGCACCGCTG ACGCATTTGG AAGACTTAAG	1440
GCAGCGGCAG AAGAAGATGC AGGCAGCTGA GTTGTTGTGT TCTGATAAGA GTCAGAGGTA	1500
ACTCCCGTTG CCGTGCTGTT AACGGTGGAG GGCAGTGTAG TCTGAGCAGT ACTCGTTGCT	1560
GCCGCGCGCG CCACCAGACA TAATAGCTGA CAGACTAACA GACTGTTTCT TTCCATGGGT	1620
CTTTTCTGCA GTCACCGTCC TTAGATCTGC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT	1680
TTGCCCCCTC CCGTGCCCTT CCTTGACCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA	1740
ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTC TATTC TGGGGGGTGG	1800

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GGTGGGGCAG CACAGCAAGG GGGAGGATTG GGAAGACAAT AGCAGGCATG CTGGGGATGC 1860
 GGTGGGCTCT ATGGGTACCC AGGTGCTGAA GAATTGACCC GGTTCCTCCT GGGCCAGAAA 1920
 GAAGCAGGCA CATCCCCTTC TCTGTGACAC ACCCTGTCCA CGCCCCCTGGT TCTTAGTTCC 1980
 AGCCCCACTC ATAGGACACT CATAGCTCAG GAGGGCTCCG CCTTCAATCC CACCCGCTAA 2040
 AGTACTTGGA GCGGTCTCTC CCTCCCTCAT CAGCCCACCA AACCAAACCT AGCCTCCAAG 2100
 AGTGGAAGA AATTAAAGCA AGATAGGCTA TTAAGTGCAG AGGGAGAGAA AATGCCTCCA 2160
 ACATGTGAGG AAGTAATGAG AGAAATCATA GAATTC 2196

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4864 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA 60
 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG 120
 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC 180
 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG 240
 CTATTGGCCA TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG 300
 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT AATCAATTAC 360
 GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT ACATAACTTA CGGTAAATGG 420
 CCCGCCTGGC TGACCGCCCA ACGACCCCGG CCCATTGACG TCAATAATGA CGTATGTTCC 480
 CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC 540
 TGCCCCCTTG GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA 600
 TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG ACCTTATGGG ACTTTCCTAC 660
 TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG GTGATGCGGT TTTGGCAGTA 720

CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT CCAAGTCTCC ACCCCATTGA	780
CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA	840
CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG	900
AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT TTGACCTCCA	960
TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA CCGTGCATTG GAACGCGGAT	1020
TCCCCGTGCC AAGAGTGACG TAAGTACCGC CTATAGAGTC TATAGGCCCA CCCCCTTGGC	1080
TTCTTATGCA TGCTATACTG TTTTGGCTT GGGGTCTATA CACCCCGCT TCCTCATGTT	1140
ATAGGTGATG GTATAGCTTA GCCTATAGGT GTGGGTTATT GACCATTATT GACCACTCCC	1200
CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC ACAACTCTCT	1260
TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA CACGGACTCT GTATTTTAC	1320
AGGATGGGGT CTCATTTATT ATTTACAAAT TCACATATAC AACACCACCG TCCCCAGTGC	1380
CCGCAGTTTT TATTAAACAT AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCGGG	1440
ACATGGGCTC TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCCTC	1500
CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA GACTTAGGCA	1560
CAGCACGATG CCCACCACCA CCAGTGTGCC GCACAAGGCC GTGGCGGTAG GGTATGTGTC	1620
TGAAAATGAG CTCGGGGAGC GGGCTTGCAC CGCTGACGCA TTTGGAAGAC TTAAGGCAGC	1680
GGCAGAAGAA GATGCAGGCA GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAACTCC	1740
CGTTGCGGTG CTGTTAACGG TGGAGGGCAG TGTAGTCTGA GCAGTACTCG TTGCTGCCGC	1800
GCGCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA TGGGTCTTTT	1860
CTGCAGTCAC CGTCCTTAGA TCTGCTGTGC CTTCTAGTTG CCAGCCATCT GTTGTTTGCC	1920
CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG GTGCCACTCC CACTGTCTT TCCTAATAAA	1980
ATGAGGAAAT TGCATCGCAT TGTCTGAGTA GGTGTCATT CATTCTGGGG GGTGGGGTGG	2040
GGCAGCACAG CAAGGGGGAG GATTGGGAAG ACAATAGCAG GCATGCTGGG GATGCGGTGG	2100
GCTCTATGGG TACCCAGGTG CTGAAGAATT GACCCGGTTC CTCCTGGGCC AGAAAGAAGC	2160
AGGCACATCC CCTTCTCTGT GACACACCCT GTCCACGCCC CTGGTTCTTA GTTCCAGCCC	2220
CACTCATAGG AACTCATAG CTCAGGAGGG CTCCGCCTTC AATCCCACCC GCTAAAGTAC	2280
TTGGAGCGGT CTCTCCCTCC CTCATCAGCC CACCAAACCA AACCTAGCCT CCAAGAGTGG	2340
GAAGAAATTA AAGCAAGATA GGCTATTAAG TGCAGAGGGA GAGAAAATGC CTCCAACATG	2400

TGAGGAAGTA	ATGAGAGAAA	TCATAGAATT	TCTTCCGCTT	CCTCGCTCAC	TGACTCGCTG	2460
CGCTCGGTCG	TTCGGCTGCG	GCGAGCGGTA	TCAGCTCACT	CAAAGGCGGT	AATACGGTTA	2520
TCCACAGAAT	CAGGGGATAA	CGCAGGAAAG	AACATGTGAG	CAAAGGCCA	GCAAAAGGCC	2580
AGGAACCGTA	AAAAGGCCGC	GTTGCTGGCG	TTTTTCCATA	GGCTCCGCC	CCCTGACGAG	2640
CATCACAAAA	ATCGACGCTC	AAGTCAGAGG	TGGCGAAACC	CGACAGGACT	ATAAAGATAC	2700
CAGGCGTTTC	CCCCTGGAAG	CTCCCTCGTG	CGCTCTCCTG	TTCCGACCCT	GCCGCTTACC	2760
GGATACCTGT	CCGCCTTTCT	CCCTTCGGGA	AGCGTGGCGC	TTTCTCAATG	CTCACGCTGT	2820
AGGTATCTCA	GTTCCGTGTA	GGTCGTTTCG	TCCAAGCTGG	GCTGTGTGCA	CGAACCCCCC	2880
GTTACAGCCG	ACCGCTGCGC	CTTATCCGGT	AACTATCGTC	TTGAGTCCAA	CCCGGTAAGA	2940
CACGACTTAT	CGCCACTGGC	AGCAGCCACT	GGTAACAGGA	TTAGCAGAGC	GAGGTATGTA	3000
GGCGGTGCTA	CAGAGTTCTT	GAAGTGGTGG	CCTAACTACG	GCTACACTAG	AAGGACAGTA	3060
TTTGGTATCT	GCGCTCTGCT	GAAGCCAGTT	ACCTTCGGAA	AAAGAGTTGG	TAGCTCTTGA	3120
TCCGGCAAAC	AAACCACCGC	TGGTAGCGGT	GGTTTTTTTG	TTTGCAAGCA	GCAGATTACG	3180
CGCAGAAAAA	AAGGATCTCA	AGAAGATCCT	TTGATCTTTT	CTACGGGGTC	TGACGCTCAG	3240
TGGAACGAAA	ACTCACGTTA	AGGGATTTTG	GTCATGAGAT	TATCAAAAAG	GATCTTCACC	3300
TAGATCCTTT	TAAATTAAAA	ATGAAGTTTT	AAATCAATCT	AAAGTATATA	TGAGTAAACT	3360
TGGTCTGACA	GTTACCAATG	CTTAATCAGT	GAGGCACCTA	TCTCAGCGAT	CTGTCTATTT	3420
CGTTCATCCA	TAGTTGCCTG	ACTCCGGGGG	GGGGGGGCGC	TGAGGTCTGC	CTCGTGAAGA	3480
AGGTGTTGCT	GACTCATACC	AGGCCTGAAT	CGCCCCATCA	TCCAGCCAGA	AAGTGAGGGA	3540
GCCACGGTTG	ATGAGAGCTT	TGTTGTAGGT	GGACCAGTTG	GTGATTTTGA	ACTTTTGCTT	3600
TGCCACGGAA	CGGTCTGCGT	TGTCGGGAAG	ATGCGTGATC	TGATCCTTCA	ACTCAGCAAA	3660
AGTTCGATTT	ATTCAACAAA	GCCGCCGTCC	CGTCAAGTCA	GCGTAATGCT	CTGCCAGTGT	3720
TACAACCAAT	TAACCAATTC	TGATTAGAAA	AACTCATCGA	GCATCAAATG	AAACTGCAAT	3780
TTATTCATAT	CAGGATTATC	AATACCATAT	TTTTGAAAAA	GCCGTTTCTG	TAATGAAGGA	3840
GAAAACTCAC	CGAGGCAGTT	CCATAGGATG	GCAAGATCCT	GGTATCGGTC	TGCGATTCCG	3900
ACTCGTCCAA	CATCAATACA	ACCTATTAAT	TTCCCTCGT	CAAAAATAAG	GTTATCAAGT	3960
GAGAAATCAC	CATGAGTGAC	GACTGAATCC	GGTGAGAATG	GCAAAAGCTT	ATGCATTTCT	4020
TTCCAGACTT	GTTCAACAGG	CCAGCCATTA	CGCTCGTCAT	CAAAATCACT	CGCATCAACC	4080

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AAACCGTTAT TCATTCTGTA TTGCGCCTGA GCGAGACGAA ATACGCGATC GCTGTTAAAA	4140
GGACAATTAC AAACAGGAAT CGAATGCAAC CGGCGCAGGA AACTGCCAG CGCATCAACA	4200
ATATTTTCAC CTGAATCAGG ATATTCCTCT AATACCTGGA ATGCTGTTTT CCCGGGGATC	4260
GCAGTGGTGA GTAACCATGC ATCATCAGGA GTACGGATAA AATGCTTGAT GGTCCGAAGA	4320
GGCATAAATT CCGTCAGCCA GTTTAGTCTG ACCATCTCAT CTGTAACATC ATTGGCAACG	4380
CTACCTTTGC CATGTTTCAG AAACAACCTCT GCGCATCGG GCTTCCCATC CAATCGATAG	4440
ATTGTCGCAC CTGATTGCCC GACATTATCG CGAGCCCATT TATACCCATA TAAATCAGCA	4500
TCCATGTTGG AATTTAATCG CGGCCTCGAG CAAGACGTTT CCCGTTGAAT ATGGCTCATA	4560
ACACCCCTTG TATTACTGTT TATGTAAGCA GACAGTTTTA TTGTTTCATGA TGATATATTT	4620
TTATCTTGTG CAATGTAACA TCAGAGATTT TGAGACACAA CGTGGCTTTC CCCCCCCCCC	4680
CATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT	4740
TAGAAAAATA AACAAATAGG GGTTCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC	4800
TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT	4860
CGTC	4864

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 41 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCACATAGAT CTGTTCCATG GTTGTGGCAA TATTATCATC G

41

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGTACAAGAT CTACCATGGC AGGAAGAAGC GGAGACAGC

39

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCACATAGAT CTGATATCGC ACTATTCTTT AGCTCCTGAC TCC

43

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATCACCATG GATGCAATGA AGAGAGGGCT CTGCTGTGTG CTGCTGCTGT GTGGAGCAGT

60

CTTCGTTTTCG CCCAGCGA

78

(2) INFORMATION FOR SEQ ID NO:19:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATCTCGCTG GCGGAAACGA AGACTGCTCC ACACAGCAGC AGCACACAGC AGAGCCCTCT 60

CTTCATTGCA TCCATGGT 78

- (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTACATGAT CAGATATCGC CCGGGCCGAG ATCTTCAGAC TTGGAGGAGG AG 52

- (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCACATTGAT CAGCTTGTGT AATTGTTAAT TTCTCTGTCC

40

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCCCGGATCC TGATCACAGA AAAATTGTGG GTCACAGTC

39

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 48 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCCAGGAAT CCACCTGTTA GCGCTTTTCT CTCTGCACCA CTCTTCTC

48

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGTACATGAT CACAGAAAAA TTGTGGGTCA CAGTC

35

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 47 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCACATTGAT CAGATATCTT ATCTTTTTTC TCTCTGCACC ACTCTTC

47

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 38 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCACCGTCC TCTATCAAAG CAGTAAGTAG TACATGCA

38

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 38 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGTACTACTT ACTGCTTTGA TAGAGGACGG TGACTGCA

38

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 40 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGTACATGAT CAACCATGAG AGTGAAGGAG AAATATCAGC

40

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCACATTGAT CAGATATCCC CATCTTATAG CAAAATCCTT TCC

43

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCACATTGAT CAGATATCCC CATCTTATAG CAAAATCCTT TCC

43

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGTACACTGC AGTCACCGTC CTATGGCAGG AAGAAGCGGA GAC

43

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCACATCAGG TACCCCATAA TAGACTGTGA CC

32

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(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGTACAAGAT CTACCATGGG ACCAGTACAA CAAATAGGTG GTAAC

45

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCACATAGAT CTTTACATTA ATCTAGCCTT CTGTCCC

37

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGTACAACCA TGGGTGGAGC TATTTCCATG AGG

33

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCTAGGTTAG CCTTCTTCTA ACCTCTTCC

29

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGTACAAGAT CTACCATGGG ATGTCTTGGG AATCAGC

37

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCACATAGAT CTGATATCGT ATGAGTCTAC TGGAAATAAG AGG

43

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGTACAGGAT CCACCATGGG TGCAGAGCG TCAGTATTAA GC

42

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CCACATGGAT CCGCCCGGGC TTACATCTCT GTACAAATTT CTACTAATGC

50

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGTACAGGAT CCCCACACGG CAAGAGCGGA GGG

33

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GGTACAGGAT CCACCATGGC TCGAGAGCG TCAGTATTAA GC

42

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCACATGGAT CCGCCCGGGC CTTTATTGTG ACGAGGGGTC GTTGC

45

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGTACAGGAT CCCCACACGG CAAGAGGCGA GGG

33

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GGTACAGGAT CCACCATGGC TGCGAGAGCG TCAGTATTAA GC

42

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTACCTCATG AGCCACATAA TACCATG

27

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(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GGTACAAGAT CTACCATGGC TTGCAATTGT CAGTTGATGC

40

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCACATAGAT CTCCATGGGA ACTAAAGGAA GACGGTCTGT TC

42

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGTACAAGAT CTACCATGAA GGCAAACCTA CTGGTCCTG

39

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCACATAGAT CTGATATCCT AATCTCAGAT GCATATTCTG CACTGC

46

(2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Arg	Ile	His	Ile	Gly	Pro	Gly	Arg	Ala	Phe	Tyr	Thr	Thr	Lys	Asn
1				5					10					15

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GAAAGAGCAG AAGACAGTGG CAATGA

26

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GGGCTTTGCT AAATGGGTGG CAAGTGGCCC GGGCATGTGG

40

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CTAACAGACT GTTCCTTTCC ATG

23

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GGAGTGGCAC CTTCCAGG

18

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

GGAGACAGCG ACGAAGACCT CCTCAAGGCA GTCAGACTCA TCAAG

45

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 71 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GATGGCTGGC AACTAGAAGG CACAGCAGAT CTGATATCGC ACTATTCTTT AGCTCCTGAC
TCCAATATTG T

60

71

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(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 119 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CTTAGATCAA CCATGAGAGT GAAGGAGAAA TATCAGCACT TGTGGAGATG GGGGTGGAGA	60
TGGGGCACCA TGCTCCTTGG GATGTTGATG ATCTGTAGTG CTACAGAAAA ATTGTGGGT	119

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CTGGCAACTA GAAGGCACAG CAGATCAGAT AGTGTCCCCA TCTTATAGCA AAATCCTTTC	60
CAAGCCCTGT CTTATTCT	78

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GATTTAGGTG AACTATAG

19

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TAATACGACT CACTATAGGG

20

(2) INFORMATION FOR SEQ ID NO:62:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CATGCCTGCA GGTCGACTCT AAATTCCG

28

(2) INFORMATION FOR SEQ ID NO:63:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

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- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

ACCCGGGGAT CCTCTAGCGC GCTTGTCTCT TGTTC

37

(2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GGGACGTGGT TTTCC

15

(2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 66 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TATGGCCACA ACCATGGCAG GAAGAAGCGG AGACAGCGAC GAAGACCTCC TCAAGGCAGT
CAGACT

60

66

(2) INFORMATION FOR SEQ ID NO:66:

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- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 90 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CTCGAGCCAT GGGCCCCTAG ACTATAGCGT GATAAGAAAT CGAGGACTGA GGTATAACA 60

TCCTCTAAGG TGGTTATAAA CTCCCGAAGG 90

(2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GCTTCGGCCA GTAACG 16

(2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 87 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TTGCATGCCT GCAGGTGGTA CATGATCAGA TATCGCCCGG GCCGAGATCT TCAGACTTGG 60
AGGAGGAGAT ATGAGGGACA ATTGGAG 87

(2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GGGGCGGAAT TTAGAGTCAA TTGATCAGCT TGTGTAATTG TTAATTTCTC TGTCCCACTC 60
CATCCAGGTC GTGTGATTC 79

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CCATCTCCAC AAGTGCTG 18

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 77 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

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- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

AGATCTAAGG ACGGTGACTG CATGTACTAC TTACTGCTTT GATAGAGGAC GGTGACTGCA 60
GAAAAGACCC ATGGAAA 77

(2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GCTTCGGCCA GTAACG 16

(2) INFORMATION FOR SEQ ID NO:73:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 62 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GGCACAGCAG ATCAGATGGG GATCTGATAT CGCACTATTC TTTAGCTCCT GACTCCTGAC 60
TC 62

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(2) INFORMATION FOR SEQ ID NO:74:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GGAATTTGAG TCATCCCCAT CTTATAGCAA AATCCTTTC AA

42

(2) INFORMATION FOR SEQ ID NO:75:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CTTAGATCCC CGCACGGCAA GAGGCGAGGG GCGGCGACTG GT

42

(2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 70 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GGCACAGCAG ATCCGCCCGG GCTTACATCT CTGTACAAAT TTCTACTAAT GCTTTTATTT 60
TTCTTCTGTC 70

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CTTAGATCCA CCATGGGTGC GAGAGCGTCA GTATTAAGCG GGGGGAGAAT TAGATCGATG 60
GGAAAAAATT 70

(2) INFORMATION FOR SEQ ID NO:78:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GGCACAGCAG ATCCGCCCGG GCTTACATCT CTGTACAAAT TTCTACTAAT GCTTTTATTT 60
TTCTTCTGTC 70

(2) INFORMATION FOR SEQ ID NO:79:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 base pairs
(B) TYPE: nucleic acid

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- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

TCACCGTCCT TAGATCACCA TGGATGCAAT GAAGAGAGGG CTCTGCTGTG TGCTGCTGCT	60
GTGTGGAGCA GTCTTCGTTT CGCCCAGCGA GATCTGCTGT GCCTTCTAGT TGCCAGCC	118

(2) INFORMATION FOR SEQ ID NO:80:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TTCGTTTCGC CCAGCGATCA CAGAAAAATT GTGGGTCACA GTC	43
---	----

(2) INFORMATION FOR SEQ ID NO:81:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

GGCACAGCAG ATCCACGTGT TAGCGCTTTT CTCTCTCCAC CAC	43
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(2) INFORMATION FOR SEQ ID NO:82:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 80 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

TCACCGTCCT TAGATCTACC ATGGGACCAG TACAACAAAT AGGTGGTAAC TATGTCCACC	60
TGCCATTAAG CCCGAGAACA	80

(2) INFORMATION FOR SEQ ID NO:83:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 44 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GGCACAGCAG ATCTTTACAT TAATCTAGCC TTCTGTCCCG GTCC	44
--	----

(2) INFORMATION FOR SEQ ID NO:84:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 80 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

TCACCGTCCT TAGATCGGTA CAACCATGGG TGGAGCTATT TCCATGAGGC AATCCAAGCC 60
 GGCTGGAGAT CTGACAGAAA 80

(2) INFORMATION FOR SEQ ID NO:85:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 85 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

GGCACAGCAG ATCACCTAGG TTAGCCTTCT TCTAACCTCT TCCTCTGACA GGCCTGACTT 60
 GCTTCCAACCT CTTCTGGGTA TCTAG 85

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 90 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

ACCGTCCTTA GATTCGACAT AGCAGAATAG GCGTTACTCG ACAGAGGAGA GCAAGAAATG 60
 GAGCCAGTAG ATCCTAGACT AGAGCCCTGG 90

(2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

GGCACAGCAG ATCCGAGATG CTGCTCCCAC CCCATCTGCT G

41

(2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

Lys	Gln	Ile	Ile	Asn	Met	Trp	Gln	Glu	Val	Gly	Lys	Ala	Met	Tyr	Ala
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:89:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

His	Glu	Asp	Ile	Ile	Ser	Leu	Trp	Asp	Gln	Ser	Leu	Lys
1				5					10			

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

Asp	Arg	Val	Ile	Glu	Val	Val	Gln	Gly	Xaa	Tyr	Arg	Ala	Ile	Arg
1				5					10					15

(2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGTACAAATA TTGGCTATTG GCCATTGCAT ACG

33

(2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CCACATCTCG AGGAACCGGG TCAATTCTTC AGCACC

36

(2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GGTACAGATA TCGGAAAGCC ACGTTGTGTC TCAAAATC

38

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

CCACATGGAT CCGTAATGCT CTGCCAGTGT TACAACC

37

(2) INFORMATION FOR SEQ ID NO:95:

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GGTACATGAT CACGTAGAAA AGATCAAAGG ATCTTCTTG

39

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

CCACATGTCG ACCCGTAAAA AGGCCGCGTT GCTGG

35

(2) INFORMATION FOR SEQ ID NO:97:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GAGCCAATAT AAATGTAC

18

(2) INFORMATION FOR SEQ ID NO:98:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CAATAGCAGG CATGC

15

(2) INFORMATION FOR SEQ ID NO:99:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

GCAAGCAGCA GATTAC

16

(2) INFORMATION FOR SEQ ID NO:100:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3547 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

GATATTGGCT ATTGGCCATT GCATACGTTG TATCCATATC ATAATATGTA CATTTATATT	60
GGCTCATGTC CAACATTACC GCCATGTTGA CATTGATTAT TGACTAGTTA TTAATAGTAA	120
TCAATTACGG GGTCAATTAGT TCATAGCCCA TATATGGAGT TCCGCGTTAC ATAACCTACG	180
GTAAATGGCC CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC AATAATGACG	240
TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC GTCAATGGGT GGAGTATTTA	300
CGGTAAACTG CCCACTTGGC AGTACATCAA GTGTATCATA TGCCAAGTAC GCCCCCTATT	360
GACGTCAATG ACGGTAAATG GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC	420
TTTCCTACTT GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT	480
TGGCAGTACA TCAATGGGCG TGGATAGCGG TTTGACTCAC GGGGATTTCC AAGTCTCCAC	540
CCCATTGACG TCAATGGGAG TTTGTTTTGG CACCAAAATC AACGGGACTT TCCAAAATGT	600
CGTAACAACCT CCGCCCCATT GACGCAAATG GGCGGTAGGC GTGTACGGTG GGAGGTCTAT	660
ATAAGCAGAG CTCGTTTAGT GAACCGTCAG ATCGCCTGGA GACGCCATCC ACGCTGTTTT	720
GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGCG GCCGGGAACG GTGCATTGGA	780
ACGCGGATTC CCCGTGCCAA GAGTGACGTA AGTACCGCCT ATAGAGTCTA TAGGCCCACC	840
CCCTTGGCTT CTTATGCATG CTATACGTGT TTTGGCTTGG GGTCTATACA CCCCCGCTTC	900
CTCATGTTAT AGGTGATGGT ATAGCTTAGC CTATAGGTGT GGGTTATTGA CCATTATTGA	960
CCACTCCCCCT ATTGGTGACG ATACTTTCCA TTACTAATCC ATAACATGGC TCTTTGCCAC	1020
AACTCTCTTT ATTGGCTATA TGCCAATACA CTGTCCTTCA GAGACTGACA CGGACTCTGT	1080
ATTTTTACAG GATGGGGTCT CATTTATTAT TTACAAATTC ACATATACAA CACCACCGTC	1140
CCCAGTGCCC GCAGTTTTTA TTAAACATAA CGTGGGATCT CCACGCGAAT CTCGGGTACG	1200
TGTTCCGGAC ATGGGCTCTT CTCCGGTAGC GGCGGAGCTT CTACATCCGA GCCCTGCTCC	1260
CATGCCTCCA GCGACTCATG GTCGCTCGGC AGCTCCTTGC TCCTAACAGT GGAGGCCAGA	1320
CTTAGGCACA GCACGATGCC CACCACCACC AGTGTGCCGC ACAAGGCCGT GGCGGTAGGG	1380
TATGTGTCTG AAAATGAGCT CGGGGAGCGG GCTTGACCG CTGACGCATT TGGAAGACTT	1440
AAGGCAGCGG CAGAAGAAGA TGCAGGCAGC TGAGTTGTTG TGTTC TGATA AGAGTCAGAG	1500
GTAACCTCCG TTGCGGTGCT GTTAACGGTG GAGGGCAGTG TAGTCTGAGC AGTACTCGTT	1560

GCTGCCGCGC	GCGCCACCAG	ACATAATAGC	TGACAGACTA	ACAGACTGTT	CCTTTCCATG	1620
GGTCTTTTCT	GCAGTCACCG	TCCTTAGATC	TGCTGTGCCT	TCTAGTTGCC	AGCCATCTGT	1680
TGTTTGCCCC	TCCCCCGTGC	CTTCCTTGAC	CCTGGAAGGT	GCCACTCCCA	CTGTCCTTTC	1740
CTAATAAAAT	GAGGAAATTG	CATCGCATTG	TCTGAGTAGG	TGTCATTCTA	TTCTGGGGGG	1800
TGGGGTGGGG	CAGCACAGCA	AGGGGGAGGA	TTGGGAAGAC	AATAGCAGGC	ATGCTGGGGA	1860
TGCGGTGGGC	TCTATGGGTA	CGGCCGCAGC	GGCCGTACCC	AGGTGCTGAA	GAATTGACCC	1920
GGTTCCTCGA	CCCGTAAAAA	GGCCGCGTTG	CTGGCGTTTT	TCCATAGGCT	CCGCCCCCCT	1980
GACGAGCATC	ACAAAAATCG	ACGCTCAAGT	CAGAGGTGGC	GAAACCCGAC	AGGACTATAA	2040
AGATAACCAGG	CGTTTCCCCC	TGGAAGCTCC	CTCGTGCCT	CTCCTGTTCC	GACCCTGCCG	2100
CTTACCGGAT	ACCTGTCCGC	CTTCTCCCT	TCGGGAAGCG	TGGCGCTTTC	TCAATGCTCA	2160
CGCTGTAGGT	ATCTCAGTTC	GGTGTAGGTC	GTTGCTCCA	AGCTGGGCTG	TGTGCACGAA	2220
CCCCCGTTC	AGCCCGACCG	CTGCGCCTTA	TCCGGTAACT	ATCGTCTTGA	GTCCAACCCG	2280
GTAAGACACG	ACTTATCGCC	ACTGGCAGCA	GCCACTGGTA	ACAGGATTAG	CAGAGCGAGG	2340
TATGTAGGCG	GTGCTACAGA	GTTCTTGAAG	TGGTGGCCTA	ACTACGGCTA	CACTAGAAGG	2400
ACAGTATTTG	GTATCTGCGC	TCTGCTGAAG	CCAGTTACCT	TCGGAAAAAG	AGTTGGTAGC	2460
TCTTGATCCG	GCAAACAAAC	CACCGCTGGT	AGCGGTGGTT	TTTTTGTTTG	CAAGCAGCAG	2520
ATTACGCGCA	GAAAAAAAGG	ATCTCAAGAA	GATCCTTTGA	TCTTTTCTAC	GTGATCCCGT	2580
AATGCTCTGC	CAGTGTTACA	ACCAATTAAC	CAATTCTGAT	TAGAAAAACT	CATCGAGCAT	2640
CAAATGAAAC	TGCAATTTAT	TCATATCAGG	ATTATCAATA	CCATATTTTT	GAAAAAGCCG	2700
TTTCTGTAAT	GAAGGAGAAA	ACTCACCGAG	GCAGTTCCAT	AGGATGGCAA	GATCCTGGTA	2760
TCGGTCTGCG	ATTCCGACTC	GTCCAACATC	AATACAACCT	ATTAATTTCC	CCTCGTCAAA	2820
AATAAGGTTA	TCAAGTGAGA	AATCACCATG	AGTGACGACT	GAATCCGGTG	AGAATGGCAA	2880
AAGCTTATGC	ATTTCTTTCC	AGACTTGTTC	AACAGGCCAG	CCATTACGCT	CGTCATCAAA	2940
ATCACTCGCA	TCAACCAAAC	CGTTATTTCAT	TCGTGATTGC	GCCTGAGCGA	GACGAAATAC	3000
GCGATCGCTG	TTAAAAGGAC	AATTACAAAC	AGGAATCGAA	TGCAACCGGC	GCAGGAACAC	3060
TGCCAGCGCA	TCAACAATAT	TTTCACCTGA	ATCAGGATAT	TCTTCTAATA	CCTGGAATGC	3120
TGTTTTCCCG	GGGATCGCAG	TGGTGAGTAA	CCATGCATCA	TCAGGAGTAC	GGATAAAATG	3180
CTTGATGGTC	GGAAGAGGCA	TAAATTCCGT	CAGCCAGTTT	AGTCTGACCA	TCTCATCTGT	3240

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AACATCATTG	GCAACGCTAC	CTTTGCCATG	TTTCAGAAAC	AACTCTGGCG	CATCGGGCTT	3300
CCCATACAAT	CGATAGATTG	TCGCACCTGA	TTGCCCCGACA	TTATCGCGAG	CCCATTTATA	3360
CCCATATAAA	TCAGCATCCA	TGTTGGAATT	TAATCGCGGC	CTCGAGCAAG	ACGTTTCCCG	3420
TTGAATATGG	CTCATAACAC	CCCTTGTAAT	ACTGTTTATG	TAAGCAGACA	GTTTTATTGT	3480
TCATGATGAT	ATATTTTTAT	CTTGTGCAAT	GTAACATCAG	AGATTTTGAG	ACACAACGTG	3540
GCTTTCC						3547

WHAT IS CLAIMED IS:

1. A polynucleotide which, upon introduction into a mammalian cell induces the co-expression in the cell of at least two gene products, comprising:
 - a first transcriptional promoter which operates in eukaryotic cells upstream from, and in transcriptional control of, a first cistron;
 - a second cistron downstream from the first cistron, under transcriptional control either of the first transcriptional promoter or under control of a second transcriptional promoter;
 - optionally, a third cistron downstream from the second cistron, under transcriptional control either of the first transcriptional promoter or under control of the second transcriptional promoter, or under control of a third transcriptional promoter; and
 - a transcriptional terminator following each of the first, second and third cistron.
2. The polynucleotide of Claim 1 wherein the first cistron encodes at least one immunogenic epitope of a pathogen or a cancer associated antigen.
3. The polynucleotide of Claim 2 wherein the pathogen is a virus.
4. The polynucleotide of Claim 3 wherein the virus is the human immunodeficiency virus (HIV).
5. The polynucleotide of Claim 2 wherein the first cistron encodes a human immunodeficiency virus (HIV) gene selected from env, gag, gag/pol, gag/protease, gag and portions of pol not encoding a functional polymerase, and pol.

6. The polynucleotide of Claim 1 wherein the second
cistron encodes a human immunodeficiency virus (HIV) REV gene if the
first cistron encodes an HIV gene, the efficient expression of which is
dependent on availability within the cell expressing the HIV gene of the
5 REV gene product.
7. The polynucleotide of Claim 6 wherein the first cistron
encodes an HIV late gene selected from env, gag and pol.
- 10 8. The polynucleotide of Claim 7 wherein the first cistron
encodes HIV gp160, HIV gp120, HIV gp41, HIV gp120 lacking a CD4
binding site and HIV env with an immunologically altered V3, the altered
V3 having an altered glycosylation pattern or substituted V3 loop tips.
- 15 9. The polynucleotide of Claim 6 wherein the third cistron
encodes a cytokine or a T-cell costimulatory element.
- 20 10. The polynucleotide of Claim 9 wherein the cytokine is
interferon, GM-CSF, or interleukin.
11. The polynucleotide of Claim 9 wherein the T-cell
costimulatory element is a gene encoding a B7 protein.
- 25 12. The polynucleotide of Claim 1 wherein the first cistron
encodes a REV-independent human immunodeficiency (HIV) epitope, the
second cistron encodes a cytokine, and the third cistron encodes a T-cell
costimulatory element, wherein each of the cistrons may also be presented
in a different order.
- 30 13. The polynucleotide of Claim 12 wherein the second
cistron encodes an interleukin, an interferon, or GM-CSF, and the third
cistron encodes a B7 protein.

14. The polynucleotide of Claim 1 wherein either of the second and third cistron is under transcriptional control of the transcriptional promoter upstream of the first cistron, a sequence is provided upstream of each of the second and third cistrons having the
5 function of an internal ribosome entry site (IRES) to effect efficient translation of the second and third cistrons on a bi- or tri-cistronic messenger RNA transcribed from the beginning of the first cistron through each of the second and third cistrons up to the transcriptional terminator following the second or third cistron.
10

15. The polynucleotide of Claim 14 wherein the IRES is selected from encephalomyocarditis virus (EMCV) IRES, swine vesicular virus IRES and poliovirus IRES.

15 16. The polynucleotide of Claim 14 wherein the first cistron encodes a human immunodeficiency virus (HIV) REV dependent gene, the second cistron encodes REV, and the third cistron encodes a T-cell costimulatory element or a cytokine, and further, wherein the first cistron is preceded by a transcriptional promoter and the second and third cistrons
20 are each preceded by an IRES and no transcriptional promoter.

17. The polynucleotide of Claim 16 wherein the first cistron encodes an HIV gp160, the first cistron is preceded by cytomegalovirus immediate early promoter, the second cistron encodes HIV REV, the
25 optional third cistron encodes an interferon, GM-CSF, an interleukin, or a B7 protein.

18. A polynucleotide which comprises contiguous nucleic acid sequences which cannot replicate in eukaryotic cells but which are
30 capable of being expressed to produce a gene product upon introduction of the polynucleotide into eukaryotic tissues in vivo, wherein the gene product either acts as an immunostimulant or as an antigen capable of generating an immune response, wherein the nucleic acid sequences encode:

a spliced REV gene;

a spliced human immunodeficiency virus (HIV)
immunogenic epitope; and
optionally, a cytokine or a T-cell recognition element.

5 19. The polynucleotide of Claim 18 wherein the HIV
immunogenic epitope selected from gag, gag-protease, or env or an
immunogenic subportion thereof; the cytokine is interleukin-12, and the T-
cell costimulatory element is a B7 protein.

10 20. The polynucleotide of Claim 19 wherein the env
immunogenic epitope is selected from HIV gp160, HIV gp120 and HIV
gp41.

15 21. The polynucleotide of Claim 19 wherein the gag
immunogenic epitope is p17, p24, or p15.

22. A polynucleotide comprising a first gene encoding an
HIV gag, gag-protease, or env immunogenic epitope, the gene containing a
REV responsive element (RRE) or having been modified to contain an
20 RRE, the gene being operatively linked with a transcriptional promoter
suitable for gene expression in a mammal, the gene being linked with an
internal ribosome entry site (IRES), and the IRES being linked with a gene
encoding a REV gene product.

25 23. The polynucleotide construct:

a) **V1Ins-revIIIB**, which has the junction sequence SEQ.ID:56:

30 5'-GGA GAC AGC GACGAA GAC CTC CTC AAG GCA GTC AGA CTC ATC AAG-3', and
SEQ.ID:57:

5'-GAT GGC TGG CAA CTA GAA GGC ACA GCA GAT CT/ GAT ATC GCA CTA
BGH **rev...**

TTC TTT AGC TCC TGA CTC CAA TAT TGT-3'

b) **VLIns-gp160IIIB**, which has the junction sequence SEQ.ID:58:

5'-CTT AGA TC/ A ACC ATG AGA GTG AAG GA GAA ATA TCA GCA CTT GTG
 5 **CMVinta** **gp160**

GAG ATG GGG GTG GAG ATG GGG CAC CAT GCT CCT TGG GAT GTT GAT GAT CTG
 TAG TGC TAC AGA AAA ATT GTG GGT-3',

10 and SEQ.ID:59:

5'-CTG GCA ACT AGA AGG CAC AGC AGA TC/ A GAT AGT GTC CCC ATC TTA
BGH **gp160**

15 TAG CAA AAT CCT TTC CAA GCC CTG TCT TAT TCT-3'

c) **pGEM-3-IRES**, which has the junction sequence SEQ.ID:62:

5'-CAT GCC TGC AGG TCG ACT CTA/ AAT TCC G...
 20 **pGEM-3 (SP6)** **IRES**

and SEQ.ID:63:

5'-A CCC GGG GAT CCT CT/ A GCG CGC TTG TCT CTT GTT CCA...
pGEM-3 (T7) **IRES**

25 d) **pGEM-3-IRES/revIIIB**, which has the junction sequence SEQ.ID:65:

5'-TAT GGC CAC AAC C/ AT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA
IRES **rev**
 30 CCT CCT CAA GGC AGT CAG ACT -3'

and SEQ.ID:66:

5'-CTC GAG CCA TGG GCC CCT/ AGA CTA TAG CGT GAT AAG AAA TCG AGG
pGEM-3 **rev**

ACT GAG GTT ATA ACA TCC TCT AAG GTG GTT ATA AAC TCC CGA AGG-3'

e) **pGEM-3-RRE/IRES/revIIIB**, which has the junction sequence SEQ.ID:68:

5'-TTG CAT GCC TGC AGG T/ GGT ACA TGA TCA GAT ATC G CCC GGG / C

5 **pGEM-3** **RRE**

CGA GAT CTT CAG ACT TGG AGG AGG AGA TAT GAG GGA CAA TTG GAG-3'

IRES-5'

¹⁰and SEQ.ID:69:

5'-GGG GCG GAA TT/ T AGA GTC A/ ATT GAT CAG CTT GTG TAA TTG TTA

RRE-3'

ATT TCT CTG TCC CAC TCC ATC CAG GTC GTG TGA TTC...-3'

15

f) **V1Jns-(tat/rev SD)**, which has the junction sequence SEQ.ID:71:

5'-AGA TCT A AGG ACG GTG ACT GCA / TGT ACT ACT TAC TGC TTT GAT

CMVintA

tat/rev SD

20

AGA GGA CGG TGA / CTG CAG AAA AGA CCC ATG GAA A-3'

CMVintA

g) **V1Jns-gp160IIIB/IRES/revIIIB (SD)**, which has the junction sequence

²⁵SEQ.ID:73:

5'-GGC ACA GCA GAT C/ AG ATG GGG ATC TGA TA TCG CAC TAT TCT TTA

BGH

rev

GCT CCT GAC TCC TGA CTC-3'

³⁰and SEQ.ID:74:

5'-GGA ATT/ TGA GTC ATC / CCC ATC TTA TAG CAA AAT CCT TTC CAA -3'

IRES

gp160

h) **V1Jns-gag-prtIIIB (SD)**, which has the junction sequence SEQ.ID:75:

5'-CTT AGA TC/ C CCG CAC GGC AAG AGG CGA GGG GCG GCG ACT GGT-3'
CMVintA ***gag (SD)***

and SEQ.ID:76:

⁵ 5'-GGC ACA GCA GAT C/ CGC CCG GGC TTA CAT CTC TGT ACA AAT TTC TAC
BGH ***pri***

TAA TGC TTT TAT TTT TCT TCT GTC...-3'

19) **V1Ins-gag-priIIB**, which has the junction sequence SEQ.ID:77:

5'-CTT AGA TC/ CAC CAT GGG TGC GAG AGC GTC AGT ATT AA GCG GGG
CMVintA ***gag***

¹⁵ GGA GAA TTA GAT CGA TGG GAA AAA ATT...-3'

and SEQ.ID:78:

5'-GGC ACA GCA GAT C/ CGC CCG GGC TTA CAT CTC TGT ACA AAT TTC TAC
BGH ***pri***

20

TAA TGC TTT TAT TTT TCT TCT GTC...-3'

j) **V1Ins-tPA**, which has the junction sequence SEQ.ID:79:

²⁵ 5'-TCA CCG TCC TTA GAT C/ ACC ATG GAT GCA ATG AAG AGA GGG CTC TGC
CMVintA **tPA leader**

TGT GTG CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC GA/ G ATC

BGH

³⁰ TGC TGT GCC TTC TAG TTG CCA GCC-3'

k) **V1Ins-tPA-gp120MN**, which has the junction sequence SEQ.ID:80:

5'-TTC GTT TCG CCC AGC GA/ TCA CAG AAA AAT TGT GGG TCA CAG TC-3'
tPA **gp120MN**

and SEQ.ID:81:

5'-GGC ACA GCA GAT C/ CAC GTG TTA GCG CTT TTC TCT CTC CAC CAC-3'
BGH **gp120MN**

5

l) V1J-SIVMAC251 p28 gag , which has the junction sequence SEQ.ID:82:

5'-TCA CCG TCC TTA GAT CT/ ACC ATG GGA CCA GTA CAA CAA ATA GGT
CMVintA **p28 gag...**

GGT AAC TAT GTC CAC CTG CCA TTA AGC CCG AGA ACA-3'
 10

and SEQ.ID:83:

5'-GGC ACA GCA GAT CT/ TTA CAT TAA TCT AGC CTT CTG TCC CGG TCC-3'
BGH **p28 gag**

15 m) V1J-SIVMAC251 nef , which has the junction sequence SEQ.ID:84:

5'-TCA CCG TCC TTA GAT C/ GGT ACA ACC ATG GGT GGA GCT ATT TCC ATG
CMVintA **nef.....**

AGG CAA TCC AAG CCG GCT GGA GAT CTG ACA GAA A-3'
 20

and SEQ.ID:85:

5'-GGC ACA GCA GAT CA/ C CTA GGT TAG CCT TCT TCT AAC CTC TTC CTC
BGH **nef....**

TGA CAG GCC TGA CTT GCT TCC AAC TCT TCT GGG TAT CTA G-3'
 25

n) V1JIns-tat/rev/env : , which has the junction sequence SEQ.ID:86:

5'-ACC GTC CTT AGA T/ TC GAC ATA GCA GAA TAG GCG TTA CTC GAC AGA
CMVintA **tat/rev/env**

30 GGA GAG CAA GAA ATG GAG CCA GTA GAT CCT AGA CTA GAG CCC TGG-3'

and SEQ.ID:87:

5'-GGC ACA GCA GAT C/ C GAG ATG CTG CTC CCA CCC CAT CTG CTG-3'.
BGH **tat/rev/env**

24. A polynucleotide which induces anti-HIV neutralizing antibody, HIV specific T-cell immune responses, or protective immune responses upon introduction into vertebrate tissue, including human tissue ⁵ in vivo, wherein the polynucleotide comprises a gene encoding a gene product selected from HIV gag, HIV gag-protease, and HIV env, the gene containing a REV responsive element (RRE), the gene being operatively linked with a transcriptional promoter suitable for gene expression in a mammal, the gene being linked with an internal ribosome entry site (IRES), ¹⁰ and the IRES being linked with a second gene, the second gene encoding a REV gene product.

25. A method for co-expression in a single cell in vivo, of at least two gene products, which comprises introducing between about 1 ng ¹⁵ and about 100 mg of the polynucleotide of Claim 1 into the tissue of the vertebrate.

26. A method for inducing immune responses in a vertebrate against HIV epitopes which comprises introducing between about 1 ng and ²⁰ about 100 mg of the polynucleotide of Claim 6 into the tissue of the vertebrate.

27. A method for inducing immune responses in a vertebrate against HIV epitopes which comprises introducing between about 1 ng and ²⁵ about 100 mg of the polynucleotide of Claim 14 into the tissue of the vertebrate.

28. A method for using a REV dependent HIV gene to induce immune responses in vivo which comprises: ³⁰
a) isolating the REV dependent HIV gene;
b) linking the isolated gene to regulatory sequences such that the gene is expressible by virtue of being operatively linked to control sequences which, when introduced into a living tissue, direct the transcription initiation and subsequent translation of the gene;

c) introducing the expressible gene into a living tissue;

and

d) introducing a gene encoding HIV REV either in trans
5 or in cis to the HIV REV dependent gene.

29. The method of Claim 28 which further comprises
boosting with additional expressible HIV gene, or boosting with a
recombinant purified HIV gene product.

10 30. The method of Claim 28 wherein the REV-dependent
HIV gene encodes a gag, or an env gene product.

31. A method for inducing immune responses against
infection or disease caused by virulent strains of HIV which comprises
15 introducing into the tissue of a vertebrate an HIV gene from a first HIV
strain such that an induced immune response neutralizes infection by the
first HIV strain but also neutralizes infection by strains heterologous to the
first strain, wherein the HIV gene encodes a conserved, REV dependent
HIV epitope and a functional REV is provided either in cis or in trans.
20

32. A vaccine for inducing immune responses against HIV
infection which comprises the polynucleotide of Claim 1 and a
pharmaceutically acceptable carrier.

25 33. A method for inducing anti-HIV immune responses in a
primate which comprises introducing the polynucleotide of Claim 1 into the
tissue of the primate and concurrently administering interleukin 12
parenterally.

30 34. The method of Claim 33 wherein the first cistron of the
polynucleotide encodes HIV gp160, the second cistron of the polynucleotide
encodes HIV REV, and the third cistron of the polynucleotide encodes B7.

35. A polynucleotide comprising:

- a) an eukaryotic transcriptional promoter;
- b) an open reading frame 3' to the transcriptional promoter encoding an immunogenic HIV epitope wherein the open reading frame has a splice donor sequence at the 5'-side of the open reading frame, a REV responsive element anywhere within the open reading frame, and a stop codon encoding the termination of translation of the open reading frame;
- c) an internal ribosome entry site (IRES) 3' to the translation stop codon of the open reading frame;
- d) an open reading frame encoding a spliced HIV REV gene at the 3' end of which is a translation stop codon;
- e) optionally, 3' to the REV translation stop codon, a second IRES, followed by an open reading frame encoding immunomodulatory or immunostimulatory genes, the genes being selected from GM-CSF, IL-12, interferon, and a B7 protein;
- f) a transcription-termination signal following the last open reading frames.

36. A method of inducing an antigen-presenting cell to stimulate cytotoxic and helper T-cell proliferation effector functions, the functions comprising lymphokine secretion specific to HIV antigens, the method comprising:

- a) exposing cells of a vertebrate in vivo to a polynucleotide, the polynucleotide comprising sequences encoding an antigenic HIV epitope, optionally, HIV REV, and sequences encoding a B7 protein.

37. The method of Claim 36 wherein the HIV epitope is selected from env, gag, and pol.

38. The method of Claim 36 wherein the polynucleotide encodes an IRES between each of the HIV epitope, the REV, and the B7 protein.

39. A polynucleotide which comprises sequences encoding:
a) an eukaryotic transcription initiation signal;
b) an HIV gene open reading frame (ORF) preceded by
an heterologous leader sequence such that expression of the HIV gene ORF
5 does not depend on availability of the HIV REV gene product;
c) a sequence which operates as an internal ribosome
entry site (IRES) 3' to the translation stop codon of the HIV ORF;
d) a sequence encoding an ORF of a T-cell costimulatory
element 3' to the IRES; and
10 e) a transcription termination signal 3' to the translation
stop codon of the T-cell costimulatory element.

40. The polynucleotide of Claim 39 wherein the HIV gene
ORF in (b) is tPAgp120 or tPAgp160.
15

41. A polynucleotide which comprises sequences encoding:
a) an eukaryotic transcription initiation signal;
b) an HIV gene open reading frame (ORF) preceded by
an heterologous leader sequence such that expression of the HIV gene ORF
20 does not depend on availability of the HIV REV gene product;
c) a sequence which operates as an internal ribosome entry site (IRES) 3' to
the translation stop codon of the HIV ORF;
d) an HIV gene open reading frame (ORF) preceded by an heterologous
leader sequence such that expression of the HIV gene ORF does not depend
25 on availability of the HIV REV gene product; and
e) a transcription termination signal 3' to the translation stop codon of the
HIV gene ORF.

42. A composition comprising multiple expression constructs
30 each of which is capable of inducing expression in mammalian tissue of
more than a single cistron encoding antigens related to disease causing
pathogens or tumors.

43. A method for immunization of a host vertebrate comprising the step of:
 introducing into direct contact with tissue of the host a non-infectious, non-integrating polynucleotide encoding at least a first and a second peptide or polypeptide, each of which is immunogenic or immunomodulatory when produced as a translation products in the host wherein the first peptide or polypeptide is encoded by a segment of the polynucleotide which is under operative control of a first transcriptional promoter and the second peptide or polypeptide is encoded by a segment of the polynucleotide under operative control of the first transcriptional promoter, in which case no transcriptional terminator is provided between the polynucleotide segment encoding the first peptide or polypeptide and the segment of polynucleotide encoding the second peptide or polypeptide, or the second peptide or polypeptide is encoded by a segment of the polynucleotide under operative control of a second transcriptional promoter, in which case a transcriptional terminator is provided between the segment of polynucleotide encoding the first peptide or polypeptide and the segment of polynucleotide encoding the second peptide or polypeptide, whereby both of the first and second peptide or polypeptide are produced within a single cell of the host, resulting in the immunization.

44. A polynucleotide construct having the elements shown in figure 2, wherein each of the first, second and third cistrons shown in the figure encode a combination of any two to three of the following:

- 1) tPA-gp120MN;
- 2) gp160III^B/IRES/REVIII^B;
- 3) gp160III^B;
- 4) REVIII^B;
- 5) tat/REV/gp160;
- 6) REV/gp160;
- 7) gp160MN;
- 8) gp160 from clinically relevant primary HIV isolates;
- 9) nef, using the gene from clinically relevant strains;
- 10) gagIII^B;

- 11) tPA-gp120^{IIIB};
- 12) gp160 with structural mutations including V3 loop substitutions from clinically relevant strains of HIV; several mutations on several constructs such as variable loop removal, Asn mutations to remove steric carbohydrate obstacles to structural, neutralizing antibody epitopes; and CD4 binding site knockout mutants;
- 13) gp41 with provision of appropriate leader sequences, as in the tPA signal peptide leader sequence;
- 14) *gag*: similar to construct from #5 above, using the gene from clinically relevant strains;
- 15) *rev*: for gp160 and *gag* dicistronics;
- 16) B7 coding sequences;
- 17) GM-CSF sequences;
- 18) Interleukin sequences;
- 19) Tumor associated antigens;
- 20) Genes encoding antigens expressed by pathogens other than HIV, such as, but not limited to, influenza virus nucleoprotein, hemagglutinin, matrix, neuraminidase, and other antigenic proteins; herpes simplex virus genes; human papillomavirus genes; tuberculosis antigens; hepatitis A, B, or C virus antigens; and combinations of these and other antigens to form at least dicistronic constructs which may be combined with multiple other polycistronic constructs to provide a cocktail composition capable of raising immune responses against all of the represented pathogens or tumor antigens;
- wherein the segments A and B of figure 2 are internal ribosome entry sites or a combination of transcription termination sequences terminating the transcription of the upstream cistron and transcriptional promoter sequences, initiating the transcription of downstream cistron.

TITLE OF THE INVENTION
COORDINATE IN VIVO GENE EXPRESSION

5 ABSTRACT OF THE INVENTION

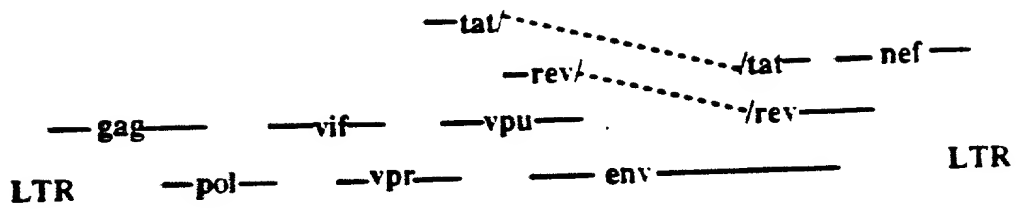
 Nucleic acids, including DNA constructs and RNA transcripts,
capable of inducing coordinate expression of two to three cistrons upon
direct introduction into animal tissues, are . bi- or tri-cistronic
polynucleotides of this invention include those encoding and co-
10 expressing HIV gene products, genes encoding antigens unrelated to
HIV, and immunostimulatory gene products, including but not limited
to GM-CSF, interleukins, interferon and members of the B7 family of
proteins which act as T-cell costimulatory elements. The methods and
polynucleotides of this invention are generally applicable to co-ordinate
15 expression in vivo of any two or more genes in a single cell.

20

25

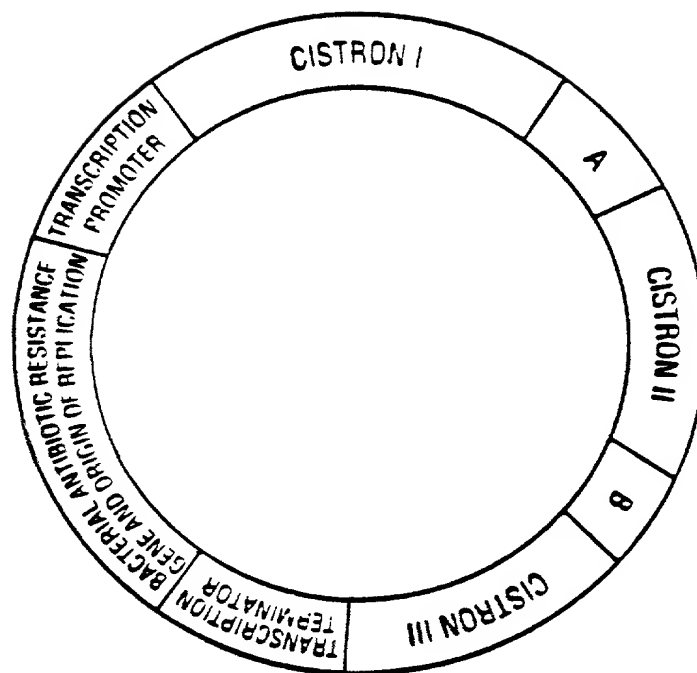
30

FIGURE 1



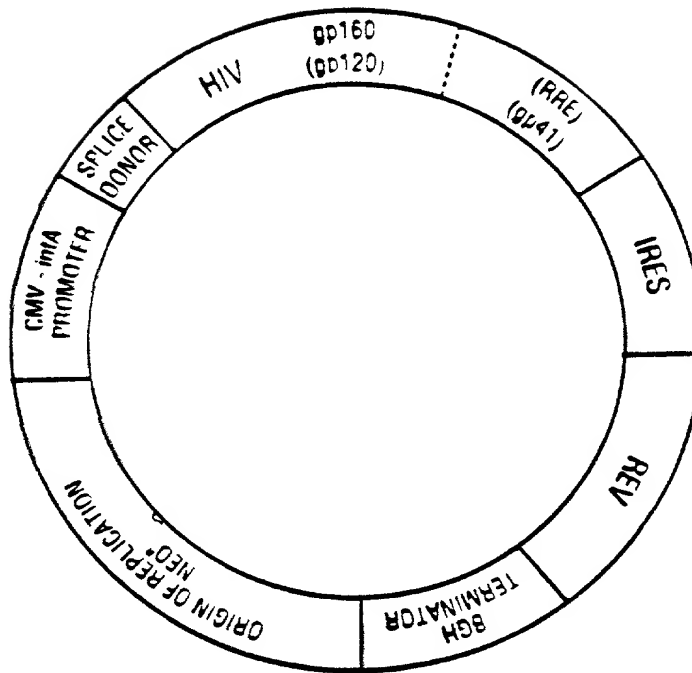
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Figure 2



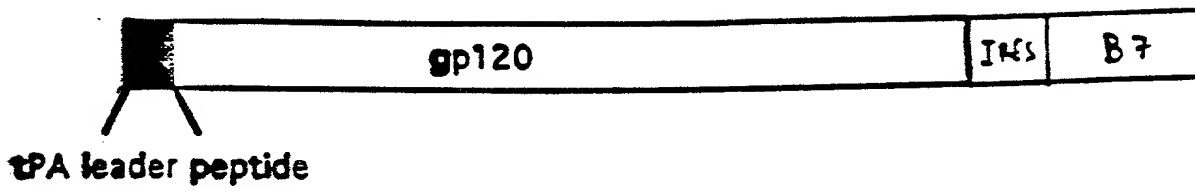
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Figure 3

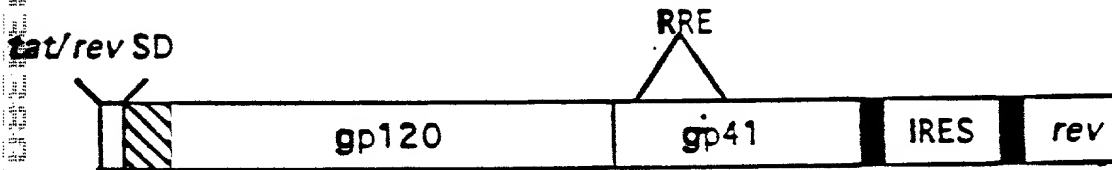


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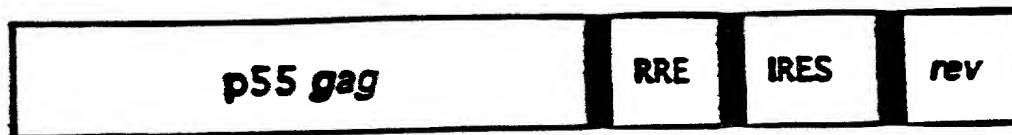
tPA-gp120 (V1Jns-tPA-gp120)



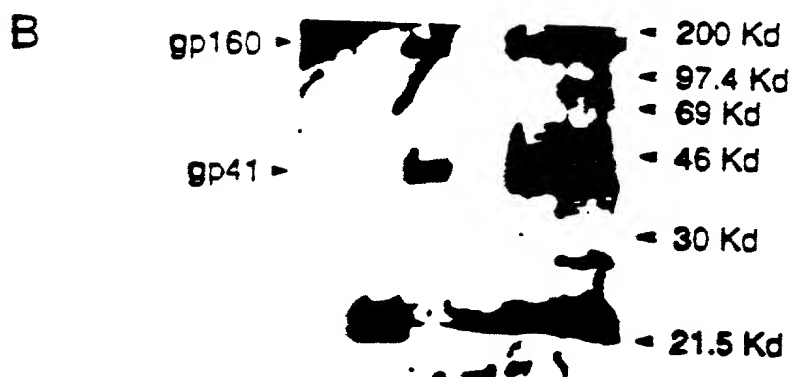
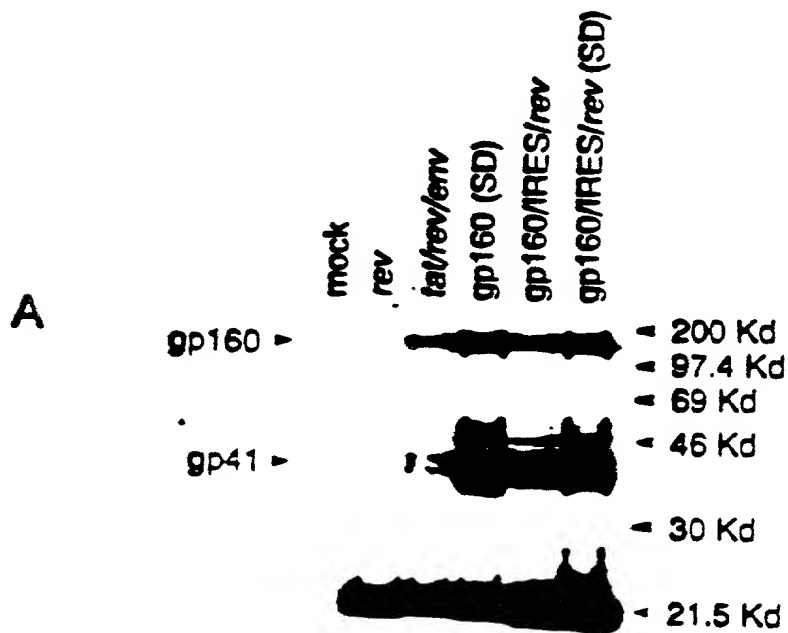
gp160/rev dicistronic construct
(V1Jns-gp160/IRES/rev/SD)



HIV gag /rev Dicistronic Construct Schematic



Anti-gp41 immunoblot Analysis of Cells Transfected
with gp160 Constructs



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Figure 6: V1J.Sequence, SEQ. ID:12:

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGGTCCCG
51 GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
101 TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTAACTATG
151 CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAATA
201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG CTATTGSCCA
251 TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG
301 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT
351 AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT
401 ACATAACTTA CGGTAAATGG CCCGCCCTGGC TGACCGCCCA ACGACCCCG
451 CCCATTGACG TCAATAATGA CGTATGTTCC CATACTAACG CCAATAGGGA
501 CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCCCTTG
551 GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA
601 TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG ACCTTATGGG
651 ACTTTCCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG
701 GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GSTTTGACTC
751 ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT
801 GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA
851 TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG
901 AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT
951 TTGACCTCCA TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA
1001 CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC
1051 CTATAGAGTC TATAGGCCCA CCCCCTTGGC TTCTTATGCA TGCTATACTG
1101 TTTTGGCTT GGGGTCTATA CACCCCGCT TCCTCATGTT ATAGGTGATG
1151 GTATAGCTTA GCCTATAGGT GTGGGTATT GACCATTATT GACCACTCCC
1201 CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC

Figure 6 (continued, p2/4)

1251 ACAACTCTCT TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA
1301 CACGGACTCT GTATTTTAC AGGATGGGGT CTCATTTATT ATTTACAAAT
1351 TCACATATAC AACACCACCG TCCCCAGTGC CCGCAGTTTT TATTAAACAT
1401 AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCGG ACATGGGCTC
1451 TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCTCT
1501 CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGGCA
1551 GACTTAGGCA CAGCAGCATG CCCACCACCA CCACTGTGCC GCACAAAGGC
1601 GTGGCGGTAG GGTATGTGTC TGAAAATGAG CTCGGGGAGC GGGCTTGCAC
1651 CGCTGACGCA TTTGGAAGAC TTAAGGCAGC GGCAGAAGAA GATGCAAGCA
1701 GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAAGTCC CGTTGGGGTG
1751 CTGTTAACGG TGGAGGGCAG TGTAGTCTGA GCACTACTCG TTGCTGCCGC
1801 GCGCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA
1851 TGGSTCTTTT CTGCAGTCAC CGTCCTTAG ATCTGCTGTG CCTTCTAGTT
1901 GCCAGCCATC TGTGTGTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA
1951 GGTGCCACTC CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA
2001 TTGTCTGAST AGGTGTCATT CTATTCTGGG GGGTGGGGTG GGGCAGCACA
2051 GCAAGGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG
2101 GGCTCTATGG GTACCCAGGT GCTGAAGAAT TGACCCGGTT CCTCCTGGGC
2151 CAGAAAGAAG CAGGCACATC CCCTTCTCTG TGACACACCC TGTCCACGCC
2201 CCTGGTTCTT AGTTCCAGCC CCACTCATAG GAACTCATA GCTCAGGAGG
2251 GCTCCGCCTT CAATCCCACC CGCTAAAGTA CTTGGAGCGG TCTCTCCCTC
2301 CCTCATCAGC CCACCAAACC AAACCTAGCC TCCAAGAGTG GGAAGAAATT
2351 AAAGCAAGAT AGGCTATTAA GTGCAGAGGG AGAGAAAATG CCTCCAACAT
2401 GTGAGGAAGT AATGAGAGAA ATCATAGAAT TTCTTCCGCT TCCTCGCTCA
2451 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GCGAGCGGT ATCAGCTCAC

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Figure 6 (continued, p3/4)

2501 TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
 2551 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCGG
 2601 CGTTGCTGGC GTTTTTCAT AGGCTCCGCC CCCCTGACGA GCATCAGAAA
 2651 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
 2701 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCGAGCCC
 2751 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
 2801 CTTTCTCAAT GCTCAGCTG TAGGTATCTC AGTTCGGTGT AGGTGCTTCG
 2851 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGGG
 2901 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
 2951 TCGCCACTGG CAGCAGCCAC TGGAACAGG ATTAGCAGAG CGAGGTATGT
 3001 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
 3051 GAAGGACAGT ATTTGGTATC TGGCTCTGTC TGAAGCCAGT TACCTTCGGA
 3101 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
 3151 TGTTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
 3201 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
 3251 AACTCAGCTT AAGGGATTTT GGTATGAGA TTATCAAAAA GGATCTTCAC
 3301 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
 3351 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
 3401 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
 3451 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG
 3501 CAATGATACC GCGAGACCCA CGCTCACCAG CTCCAGATTT ATCAGCAATA
 3551 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC
 3601 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT
 3651 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG
 3701 GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG

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Figure 6 (continued, p4/4)

3751 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT
3801 CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
3851 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT
3901 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
3951 AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT
4001 ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTG
4051 TTCGSSGCGA AAACCTCTCA GGATCTTACC GCTGTTGAGA TCCAGTTGGA
4101 TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTGACG
4151 AGCGTTTCTG GGTGAGCAA AACAGGAAGG CAAAATGCCG CAAAAAGGG
4201 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
4251 ATTATTGAAG CATTTATCAG GGTATTGTC TCATGAGCGG ATACATATTT
4301 GAATGTATTT AGAAAAATAA ACAATAGGG GTTCCGCGCA CATTTCCCCG
4351 AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT
4401 ATAAAAATAG GCGTATCAG AGGCCCTTTC GTC

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Figure 7: V1Jneo Sequence, SEQ. ID:14:

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCC
51 GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAACCCC
101 TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG
151 CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAATAA
201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG CTATTGSCCA
251 TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG
301 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT
351 AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGGGTT
401 ACATAACTTA CGGTAAATGG CCCGCCCTGGC TGACCGCCCA ACGACCCCCG
451 CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA
501 CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCCACTTG
551 GCASTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA
601 TGACGGTAA TGGCCCCGCT GGCATTATGC CCASTACATG ACCTTATGGG
651 ACTTTCCTAC TTGGCASTAC ATCTACGTAT TAGTCATCGC TATTACCATG
701 GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTGTGACTC
751 ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT
801 GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA
851 TTGACGCAA TGGGCGGTAG GCGTGACGG TGGGAGGTCT ATATAAGCAG
901 AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT
951 TTGACCTCCA TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA
1001 CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC
1051 CTATAGAGTC TATAGGCCCA CCCCCTTGGC TTCTTATGCA TGCTATACTG
1101 TTTTGGCTT GGGGTCTATA CACCCCCGCT TCCTCATGTT ATAGGTGATG
1151 GTATAGCTTA GCCTATAGGT GTGGGTATT GACCATTATT GACCACTCCC

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Figure 7 (continued, p2/4)

1201 CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC
 1251 ACAACTCTCT TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA
 1301 CACGGACTCT GTATTTTAC AGGATGGGGT CTCATTTATT ATTTACAAAT
 1351 TCACATATAC AACACCACCG TCCCCAGTGC CCGCAGTTTT TATTAACAT
 1401 AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCCGG ACATGGGCTC
 1451 TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGGCTC
 1501 CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA
 1551 GACTTAGGCA CAGCAGATG CCCACCACCA CCAGTGTGCC GCACAAAGCC
 1601 GTGGCGGTAG GGTATGTGTC TGAAAATGAG CTCGGGAGAG GGGCTTGCAC
 1651 CGCTGACGCA TTTGGAAGAC TTAAGGCAGC GGCAGAAAGAA GATGCAGGCA
 1701 GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAACCTC CGTTGGGCTG
 1751 CTGTTAAGCG TGGAGGGCAG TGTAGTCTGA GCAGTACTCG TTGCTGCCGC
 1801 GCGCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA
 1851 TGGGTCTTTT CTGCACTCAC CGTCCTTAG ATCTGCTGTG CCTTCTAGTT
 1901 GCCAGCCATC TGTGTGTTGC CCTCCCCCG TGCCCTTCCTT GACCCTGGAA
 1951 GGTGCCACTC CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA
 2001 TTGTCTGAGT AGGTGTCATT CTATTCTGGG GGGTGGGGTG GGGCAGCACA
 2051 GCAAGGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG
 2101 GGCTCTATGG GTACCCAGGT GCTGAAGAAT TGACCCGGTT CCTCCTGGGC
 2151 CAGAAAGAAG CAGGCACATC CCCTTCTCTG TGACACACCC TGTCCACGCC
 2201 CCTGGTTCTT AGTTCCAGCC CCACTCATAG GACTCATA GCTCAGGAGG
 2251 GCTCCGCCTT CAATCCCACC CGCTAAAGTA CTTGGAGCGG TCTCTCCCTC
 2301 CCTCATCAGC CCACCAAACC AACCTAGCC TCCAAGAGTG GGAAGAAATT
 2351 AAAGCAAGAT AGGCTATTAA GTGCAGAGGG AGAGAAAATG CCTCCAACAT
 2401 GTGAGGAAGT AATGAGAGAA ATCATAGAAT TTCTTCGCT TCCTCGCTCA

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Figure 7 (continued, p3/4)

2451 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GCGAGCGGT ATCAGCTCAC
 2501 TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
 2551 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCGG
 2601 CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
 2651 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
 2701 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
 2751 TGCCGCTTAC CGGATACCTG TCCGCTTTTC TCCCTTCGGG AAGCGTGGCG
 2801 CTTTCTCAAT GCTCAGCTG TAGGTATCTC AGTTCGGTGT AGGTGGTTCG
 2851 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGGG
 2901 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
 2951 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT
 3001 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
 3051 GAAGGACAGT ATTTGSTATC TCGCTCTGC TGAAGCCAGT TACCTTCGGA
 3101 AAAAGAGTTG GTAGCTCTTG ATCCGGCAA CAAACCACCG CTGGTAGCGG
 3151 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
 3201 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
 3251 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC
 3301 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
 3351 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
 3401 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCGGGG
 3451 GGGGGGGGCG CTGAGGTCTG CCTCGTGAAG AAGGTGTTGC TGACTCATAC
 3501 CAGGCCTGAA TCGCCCCATC ATCCAGCCAG AAAGTGAGGG AGCCACGGTT
 3551 GATGAGAGCT TTGTTGTAGG TGGACCAGTT GGTGATTTTG AACTTTTGCT
 3601 TTGCCACGGA ACGGTCTGCG TTGTCGGGAA GATGCGTGAT CTGATCCTTC
 3651 AACTCAGCAA AAGTTCGATT TATTCAACAA AGCCGCCGTC CCGTCAAGTC

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Figure 7 (continued, p4/4)

3701 AGCGTAATGC TCTGCCAGTG TTACAACCAA TTAACCAATT CTGATTAGAA
 3751 AAACATCATCG AGCATCAAAT GAAACTGCAA TTTATTCATA TCAGGATTAT
 3801 CAATACCATA TTTTGTGAAA AGCCGTTTCT GTAATGAAGG AGAAACTCA
 3851 CCGAGGCAGT TCCATAGGAT GGCAAGATCC TGGTATCGGT CTGCGATTCC
 3901 GACTCGTCCA ACATCAATAC AACCTATTAA TTTCCCTCG TCAAAATTA
 3951 GGTTATCAAG TGAGAAATCA CCATGAGTGA CGACTGAATC CGGTGAGAA
 4001 GGCAAAAGCT TATGCATTTT TTTCCAGACT TGTTCACAG GCCAGCCATT
 4051 ACGCTCGTCA TCAAAATCAC TCGCATCAAC CAAACCGTTA TTCATTCTG
 4101 ATTGCGCCTG AGCGAGACGA AATACGCGAT CGCTGTAAA AGGACAATTA
 4151 CAAACAGGAA TCGAATGCAA CCGGCGCAGG AACACTGCCA GCGCATCAAC
 4201 AATATTTTCA CCTGAATCAG GATATTCTTC TAATACCTGG AATGCTGTTT
 4251 TCCCGGGGAT CGCAGTGGTG AGTAACCATG CATCATCAGG AGTACGGATA
 4301 AAATGCTTGA TGGTCGGGAG AGGCATAAAT TCCGTCAGCC AGTTTASTCT
 4351 GACCATCTCA TCTGTAAAT CATTTGGCAAC GCTACCTTTG CCATGTTTCA
 4401 GAAACAACCTC TGGCGCATCG GGCTTCCCAT ACAATCGATA GATTGTGCGA
 4451 CCTGATTGCC CGACATTATC GCGAGCCCAT TTATACCCAT ATAAATCAGC
 4501 ATCCATGTTG GAATTTAATC GCGGCCTCGA GCAAGACGTT TCCCGTTGAA
 4551 TATGGCTCAT AACACCCCTT GTATTACTGT TTATGTAAGC AGACAGTTTT
 4601 ATTGTTTCATG ATGATATATT TTTATCTTGT GCAATGTAAC ATCAGAGATT
 4651 TTGAGACACA ACGTGGCTTT CCCCCCCCCC CCATTATTGA AGCATTTATC
 4701 AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT
 4751 AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT
 4801 CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT AGGCGTATCA
 4851 CGAGGCCCTT TCGTC

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Figure 8: CMVintaBSH Sequence, SEQ. ID:13:

```

1  ATTGGCTATT GGCCATTGCA TACGTTGTAT CCATATCATA ATATGTACAT
51  TTATATTGGC TCATGTCCAA CATTACCGCC ATGTTGACAT TGATTATTGA
101 CTAGTTATTA ATAGTAATCA ATTACGGGGT CATTAGTTCA TAGCCCATAT
151 ATGGAGTTCC GCGTTACATA ACTTACGGTA AATGGCCCCG CTGGGTGACC
201 GCCCAACGAC CCCCGCCCAT TGACGTCAAT AATGACGTAT GTTCCCATAG
251 TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA GTATTTACGG
301 TAAACTGCCC ACTTGGCACT ACATCAAGTG TATCATATGC CAAGTACGCG
351 CCTATTGAC GTCATGACG GTAAATGGCC CGCCTGSCAT TATGCCCACT
401 ACATGACCTT ATGGGACTTT CCTACTTGGC AGTACATCTA CGTATTAGTC
451 ATCGCTATTA CCATGGTGAT GCGGTTTTGG CAGTACATCA ATGGGCGTGG
501 ATAGCGGTTT GACTCACGGG GATTTCCAAG TCTCCACCCC ATTGACGTCA
551 ATGGGAGTTT GTTTTGGCAC CAAATCAAC GGGACTTTCC AAAATGTGCT
601 AACAACTCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA
651 GGTCTATATA AGCAGAGCTC GTTTAGTGAA CCGTCAGATC GCCTGGAGAC
701 GCCATCCACG CTGTTTTGAC CTCCATAGAA GACACCGGGA CCGATCCAGC
751 CTCGCGGGCC GGGAACGGTG CATTGGAACG CGGATTCCCC GTGCCAAGAG
801 TGACGTAAGT ACCGCTATA GAGTCTATAG GCCCACCCCC TTGCTTCTCT
851 ATGCATGCTA TACTGTTTTT GGCTTGGGGT CTATACACCC CCGCTTCTCT
901 ATGTTATAGG TGATGGTATA GCTTAGCCTA TAGGTGTGGG TTATTGACCA
951 TTATTGACCA CTCCCCTATT GGTGACGATA CTTTCCATTA CTAATCCATA
1001 ACATGGCTCT TTGCCACAAC TCTCTTTATT GGCTATATGC CAATACACTG
1051 TCCTTCAGAG ACTGACACGG ACTCTGTATT TTTACAGGAT GGGGTCTCAT
1101 TTATTATTTA CAAATTCACA TATACAACAC CACCGTCCCC AGTGCCCGCA
1151 GTTTTTATTA AACATAACGT GGGATCTCCA CGCGAATCTC GGGTACGTGT
1201 TCCGGACATG GGCTCTTCTC CGGTAGCGGC GGAGCTTCTA CATCCGAGCC

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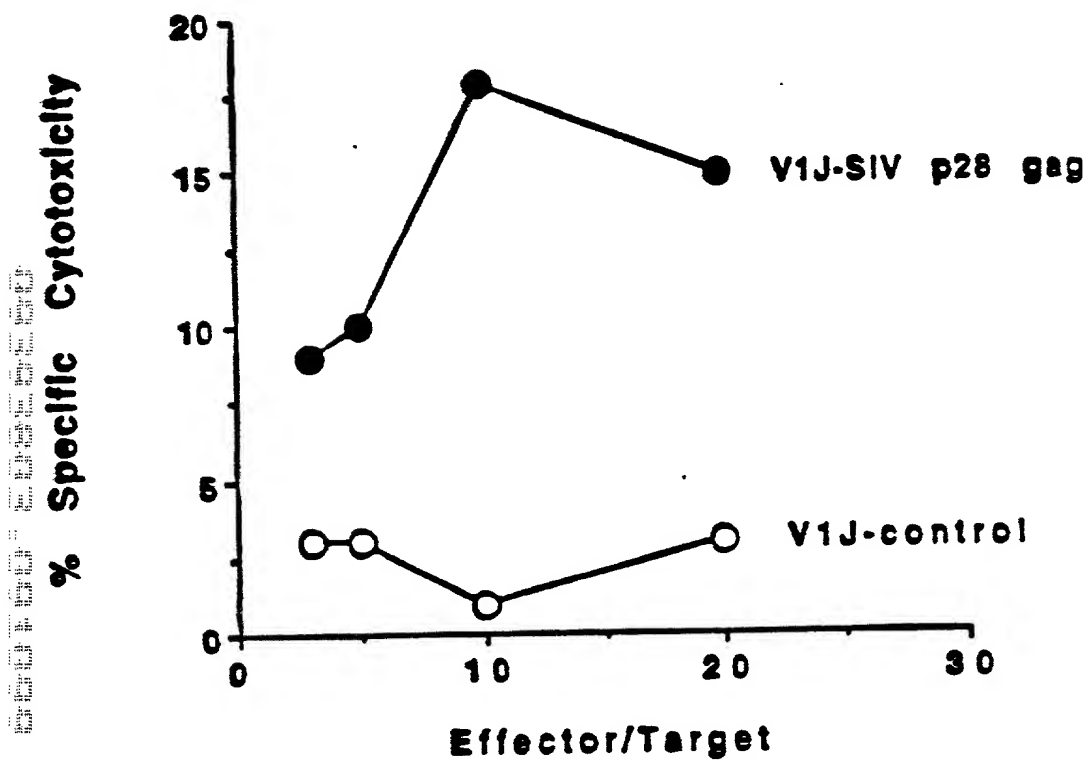
Figure 8 (continued, p2/2)

1251 CTGCTCCCAT GCCTCCAGCG ACTCATGGTC GCTCGGCAGC TCCTTGCTCC
1301 TAACAGTGGA GGCCAGACTT AGGCACAGCA CGATGCCCCAC CACCACCACT
1351 GTGCCGCACA AGGCCGTGGC GGTAGGGTAT GTGTCTGAAA ATGAGCTCGG
1401 GGAGCGGGCT TGCACCGCTG ACGCATTGAG AAGACTTAAG GCAGCGGCAG
1451 AAGAAGATGC AGGCAGCTGA GTTGTGTGT TCTGATAAGA GTCAGAGGTA
1501 ACTCCCGTTG CGGTGCTGTT AACGGTGGAG GGCAGTGTAG TCTGAGCACT
1551 ACTCGTTGCT GCCGCGCGCG CCACCAGACA TAATAGCTGA CAGACTAACA
1601 GACTGTTTCT TTCCATGGGT CTTTTCTGCA GTCACCGTCC TTAGATCTG
1651 CTGTGCCTTC TAGTTGCCAG CCATCTGTTG TTTGCCCCCTC CCCCCTGCTC
1701 TCCTTGACCC TGGAAAGGTGC CACTCCCCACT GTCCTTTTCT AATAAATGA
1751 GGAAATTGCA TCGCATTGTC TGAGTAGGTG TCATTCTATT CTGGGGGGTG
1801 GGGTGGGGCA GCACAGCAAAG GGGGAGGATT GGGAAAGACAA TAGCAGGCAT
1851 GCTGGGGATG CGGTGGGGCTC TATGGGTACC CAGGTGCTGA AGAATTGACC
1901 CGGTTCTCTC TGGGCCAGAA AGAAGCAGGC ACATCCCCCTT CTCTGTGACA
1951 CACCCTGTCC ACGCCCCCTGG TTCTTAGTTC CAGCCCCACT CATAGGACAC
2001 TCATAGCTCA GGAGGGCTCC GCCTTCAATC CCACCCGCTA AAGTACTTGG
2051 AGCGGTCTCT CCCTCCCTCA TCAGCCCCACC AAACCAAACC TAGCCTCCAA
2101 GAGTGGGAAG AAATTAAAGC AAGATAGGCT ATTAAGTGCA GAGGGAGAGA
2151 AAATGCCTCC AACATGTGAG GAAGTAATGA GAGAAATCAT AGAATTC

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Figure 9

**Primary CTL Generation by V1J-SIV p28 gag
DNA Vaccination**

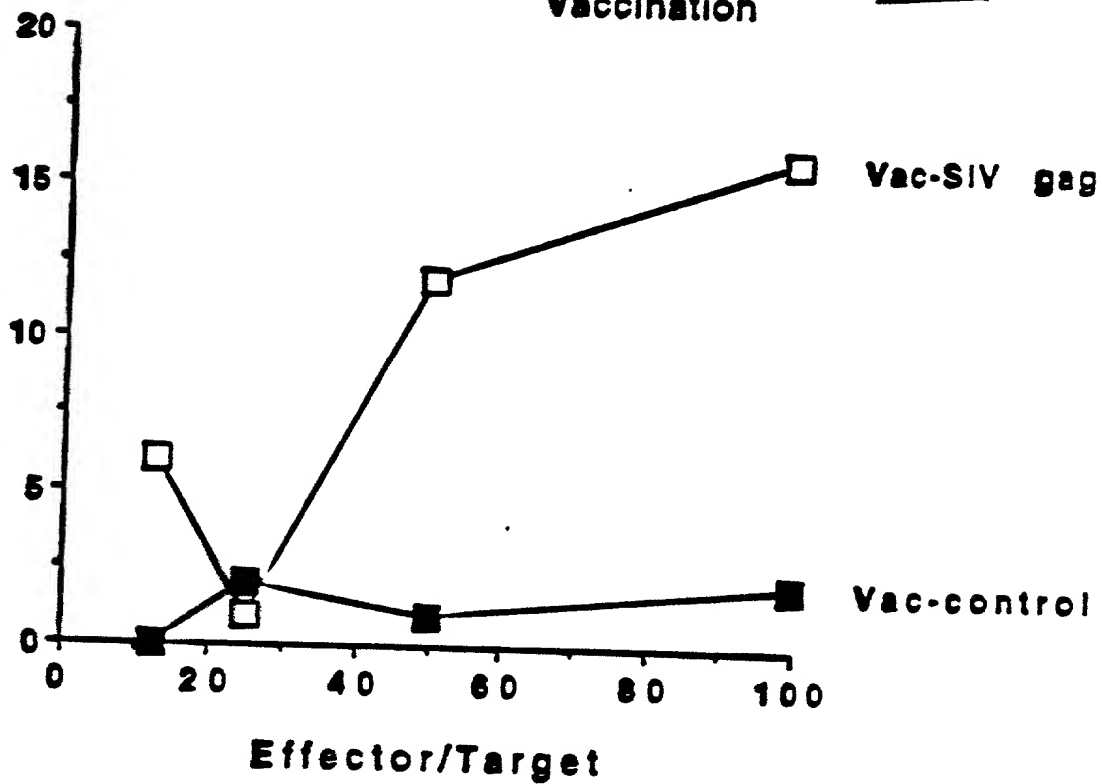


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Figure 10

Primary CTL Generation by Vaccinia-SIV gag Vaccination

% Specific Cytotoxicity



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FIGURE 11, VIR SEQUENCE, SEQ.ID:100:

```

1  GATATTGG CTATTGGCCA
251 TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG
301 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT
351 AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCGGCGTT
401 ACATAACTTA CGGTAAATGG CCGGCCTGGC TGACCGCCCA ACGACCCCGG
451 CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA
501 CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG
551 GCASTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA
601 TGACGGTAAA TGCCCCCGCT GGCATTATGC CCASTACATG ACCTTATGGG
651 ACTTTCCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG
701 GTGATGGGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC
751 ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT
801 GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCGCCCCCA
851 TTGACGCAAA TGSSCGGTAG GCGGTACGG TGCGAGGTCT ATATAAGCAG
901 AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT
951 TTGACCTCCA TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA
1001 CGGTGCATTG GAACGGGGAT TCCCCGTGCC AAGAGTGACG TAASTACCGC
1051 CTATAGAGTC TATAGCCCCA CCCCCCTGGC TTCTTATGCA TGCTATACTG
1101 TTTTGGGCTT GGGGTCTATA CACCCCGCT TCCTCATGTT ATAGGTGATG
1151 GTATAGCTTA GCCTATAGGT GTGGGTATT GACCATTATT GACCACTCCC
1201 CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC
1251 ACAACTCTCT TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA
1301 CACGGACTCT GTATTTTAC AGGATGGGGT CTCATTATT ATTTACAAAT
1351 TCACATATAC AACACCACCG TCCCCAGTGC CCGCAGTTT TATTAAACAT

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Figure 11 (continued, p2/3)

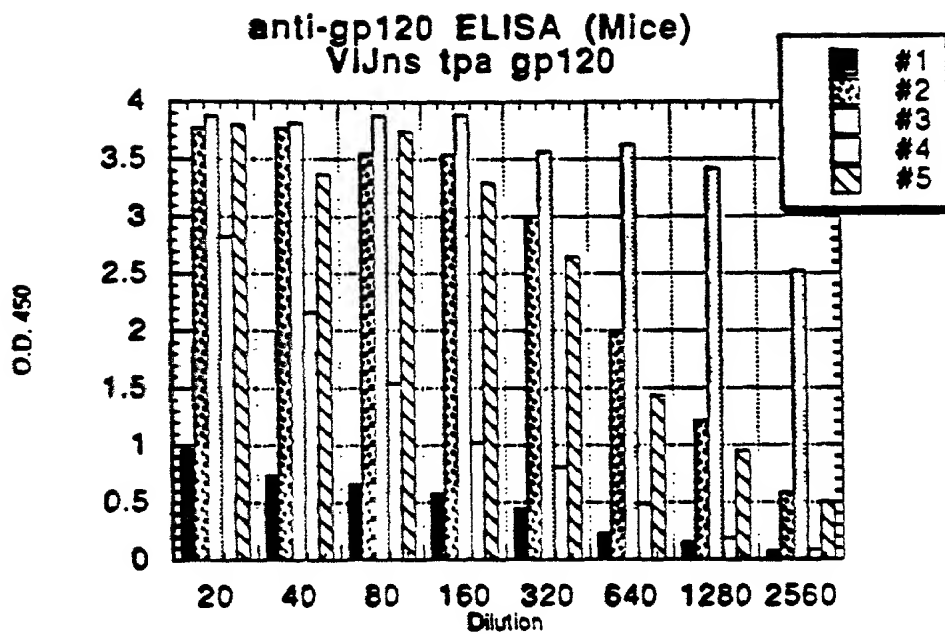
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 1451 TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCCTC
 1501 CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA
 1551 GACTTAGGCA CAGCAGGATG CCCACCACCA CCAGTGTGCC GCACAAGGCC
 1601 GTGGCGGTAG GGTATGTGTC TGAAAATGAG CTCGGGGAGC GGGCTTGCCAC
 1651 CGCTGACGCA TTTGGAAGAC TTAAGGCAGC GGCAGAAGAA GATGCAGGCA
 1701 GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAAGTCC CGTTGCGGTG
 1751 CTGTTAACGG TGGAGGGCAG TGTASTCTGA GCAGTACTCG TTGCTGCCCG
 1801 GCGCGGCCAC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCGA
 1851 TGGGTCTTTT CTGCASTCAC CGTCCTTAG ATCTGCTGTG CTTTCTAGTT
 1901 GCCAGCCATC TGTGTTTGGC CCTCCCCCG TGCCTTCCTT GACCCTGGAA
 1951 GGTGCCACTC CCACTGTCTT TTCTAATAA AATGAGGAAA TTGCATCGCA
 2001 TTGTCTGAST AGGTGTCATT CTATTCTGGS GGGTGGGGTG GGGCAGCACA
 2051 GCAAAGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGGGGTG
 2101 GCTCTATGG GTAC GCGCGCAGCGGC GTACCCAGGT GCTGAAGAA
 TGACCCGGTT CCTCGACCCGT AAAAAGGCGG
 2601 CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
 2651 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
 2701 CCAGSCGTTT CCCCCGGA GCTCCCTCGT GCGCTCTCCT GTTCGGACCC
 2751 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
 2801 CTTTCTCAAT GCTCAGCTG TAGGTATCTC AGTTGGGTGT AGGTGCTTCG
 2851 CTCCAAGCTG GGCTGTGTGC ACCAACCCCC CATTACAGCC GACCGCTGCG
 2901 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
 2951 TCGCCACTGG CAGCAGCCAC TGCTAACAGG ATTACAGAG CGAGGTATGT
 3001 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA

Figure 11 (continued, p3/3)

3051 GAAGGACAGT ATTTGGTATC TGGGCTCTGC TGAAGCCAGT TACCTTCGGA
 3101 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
 3151 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
 3201 AAGAAGATCC TTTGATCTTT TCTACGTGATCC CGTAATGC TCTGCCASTG
 TTACAACCAA TTAACCAATT CTGATTAGAA
 3751 AAACATCATC AGCATCAAAT GAAACTGCAA TTTATTGATA TCAGGATTAT
 3801 CAATACCATA TTTTGGAAAA AGCCGTTTCT GTAATGAAGG AGAAAACCTCA
 3851 CCGAGGCGAGT TCCATAGGAT GGCAAGATCC TGGTATCGGT CTGCGATTCC
 3901 GACTCGTCCA ACATCAATAC AACCTATTAA TTTCCCTCTG TCAAAAATAA
 3951 GGTATCAAG TGAGAAATCA CCATGAGTGA CGACTGAATC CGGTGAGAAAT
 4001 GGCAAAAGCT TATGCATTTC TTTCCAGACT TGTTCACAG GGCAGCCATT
 4051 ACGCTCGTCA TCAAAATCAC TCGCATCAAC CAAACCGTTA TTCATTGCTG
 4101 ATTGGCGCTG AGCGAGACGA AATACGCGAT CGCTGTTAA AGGACAATTA
 4151 CAAACAGGAA TCGAATGCAA CCGGCGCAGG AACACTGCCA GCGCATCAAC
 4201 AATATTTTCA CCTGAATCAG GATATTCTTC TAATACCTGG AATGCTGTTT
 4251 TCCCGGGGAT CGCAGTGGTG AGTAACCATG CATCATCAGG AGTACGGATA
 4301 AAATGCTTGA TGSTCGGAAG AGGCATAAAT TCCGTCAGCC AGTTTAGTCT
 4351 GACCATCTCA TCTGTAACAT CATTGGCAAC GCTACCTTTG CCATGTTTCA
 4401 GAAACAACTC TGGCGCATCG GGCTTCCCAT ACAATCGATA GATTGTGCGA
 4451 CCTGATTGCC CGACATTATC GCGAGCCCAT TTATACCCAT ATAAATCAGC
 4501 ATCCATGTTG GAATTTAATC GCGGCTCGA CCAAGACGTT TCCCGTTGAA
 4551 TATGGCTCAT AACACCCCTT GTATTACTGT TTATGTAAGC AGACAGTTTT
 4601 ATTGTTGATG ATGATATATT TTTATCTTGT GCAATGTAAC ATCAGAGATT
 4651 TTGAGACACA ACGTGGCTTT CC

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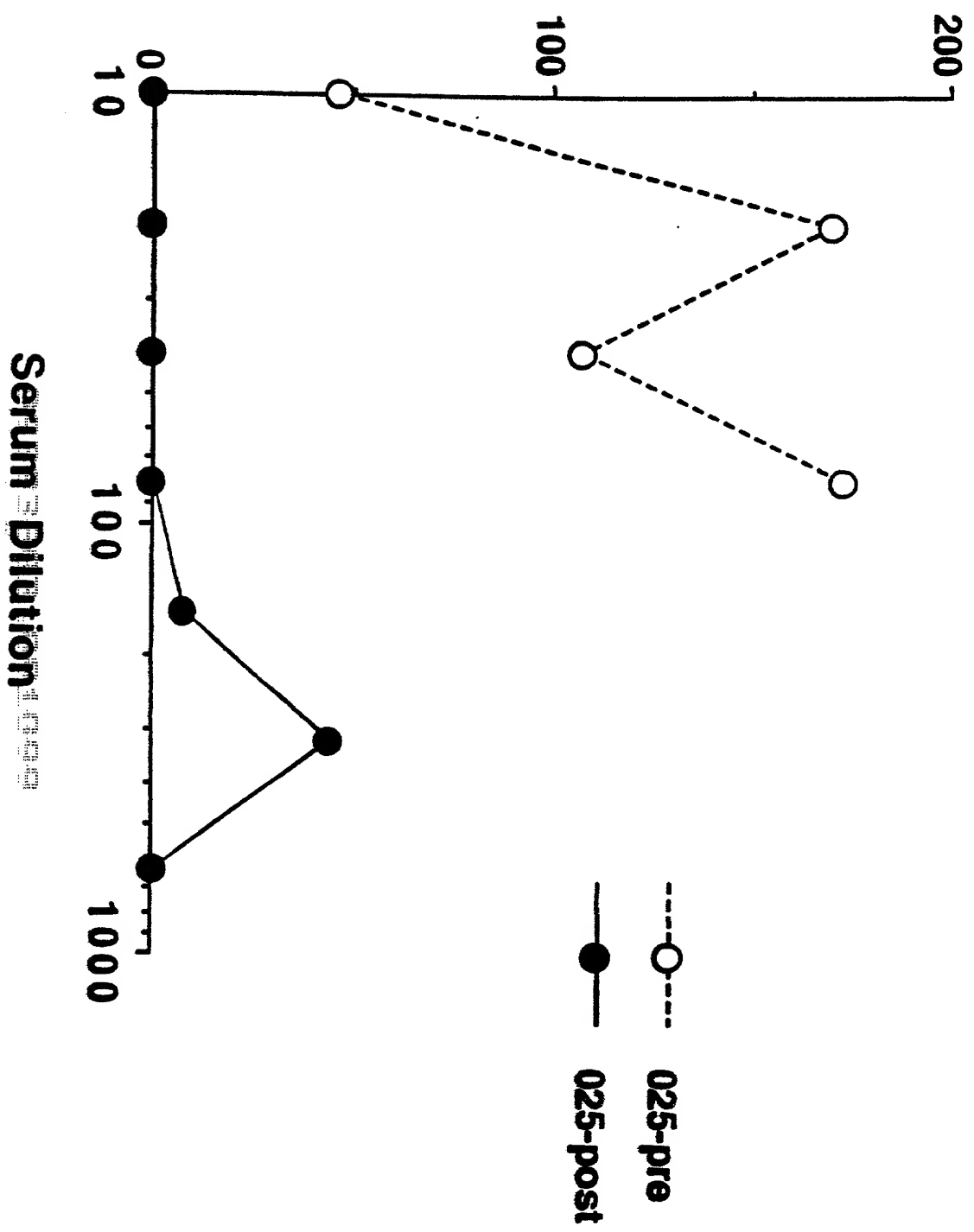
Figure 12



HIV MN Neutralization by Serum from tPA-gp120 DNA Immunized African Green Monkeys

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Figure 12A

p24 gag (ng/ml)



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Figure 12B

p24 gag (ng/ml)

HIV MN Neutralization by Serum from tPA-gp120 DNA Immunized African Green Monkeys

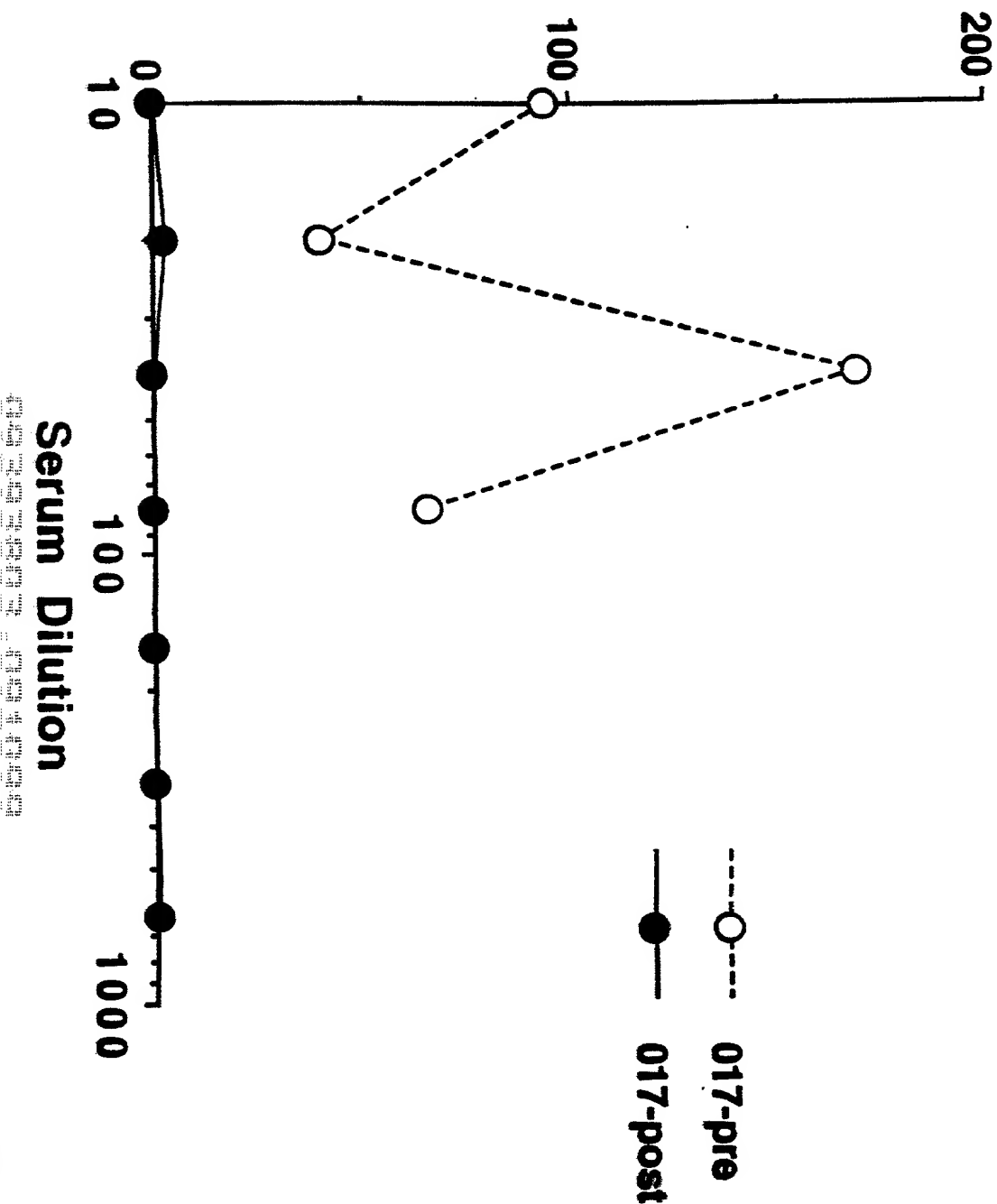
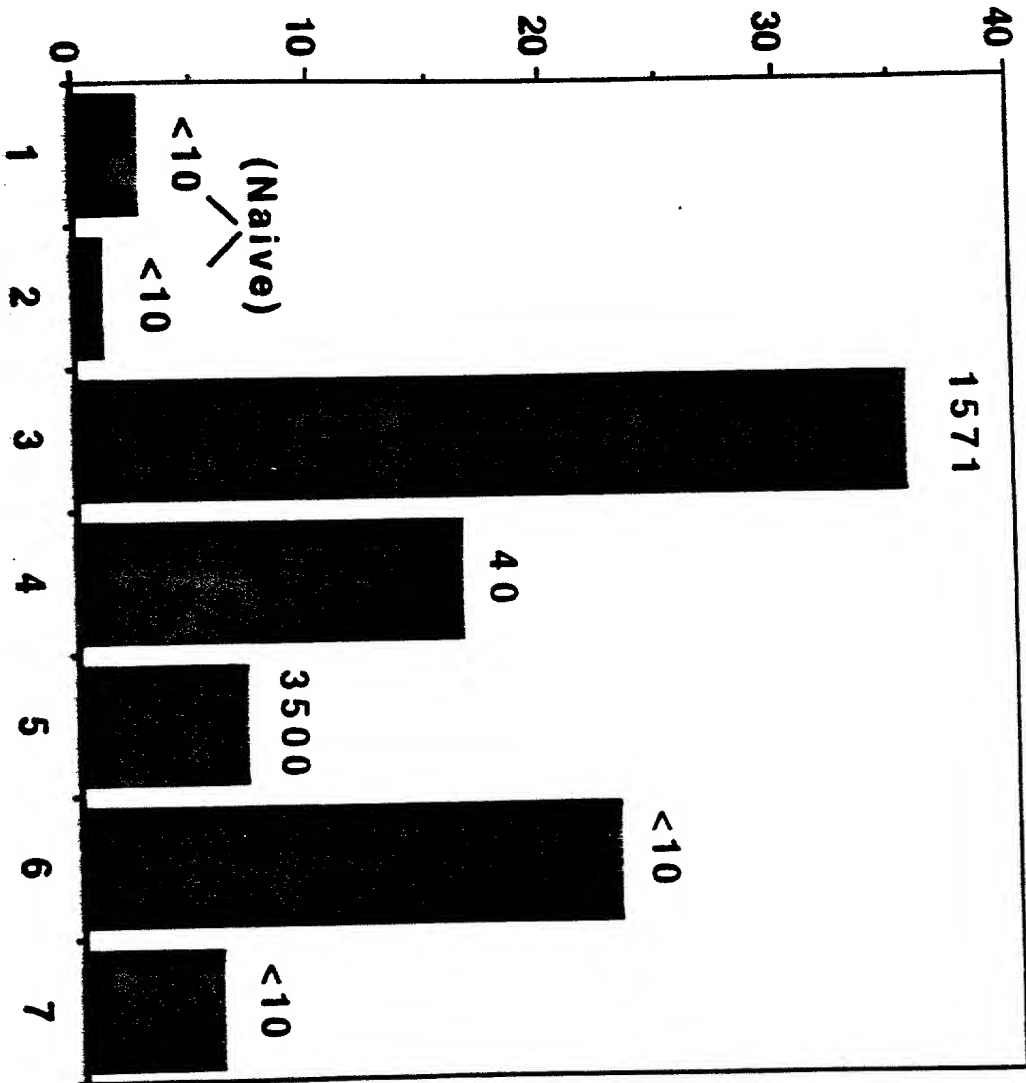


Figure 13

Stimulation Index

T Cell Proliferation by gp120 DNA Immunized Mice- 6 months post
(1.6 ug DNA/mouse; 2X)



NOTE:

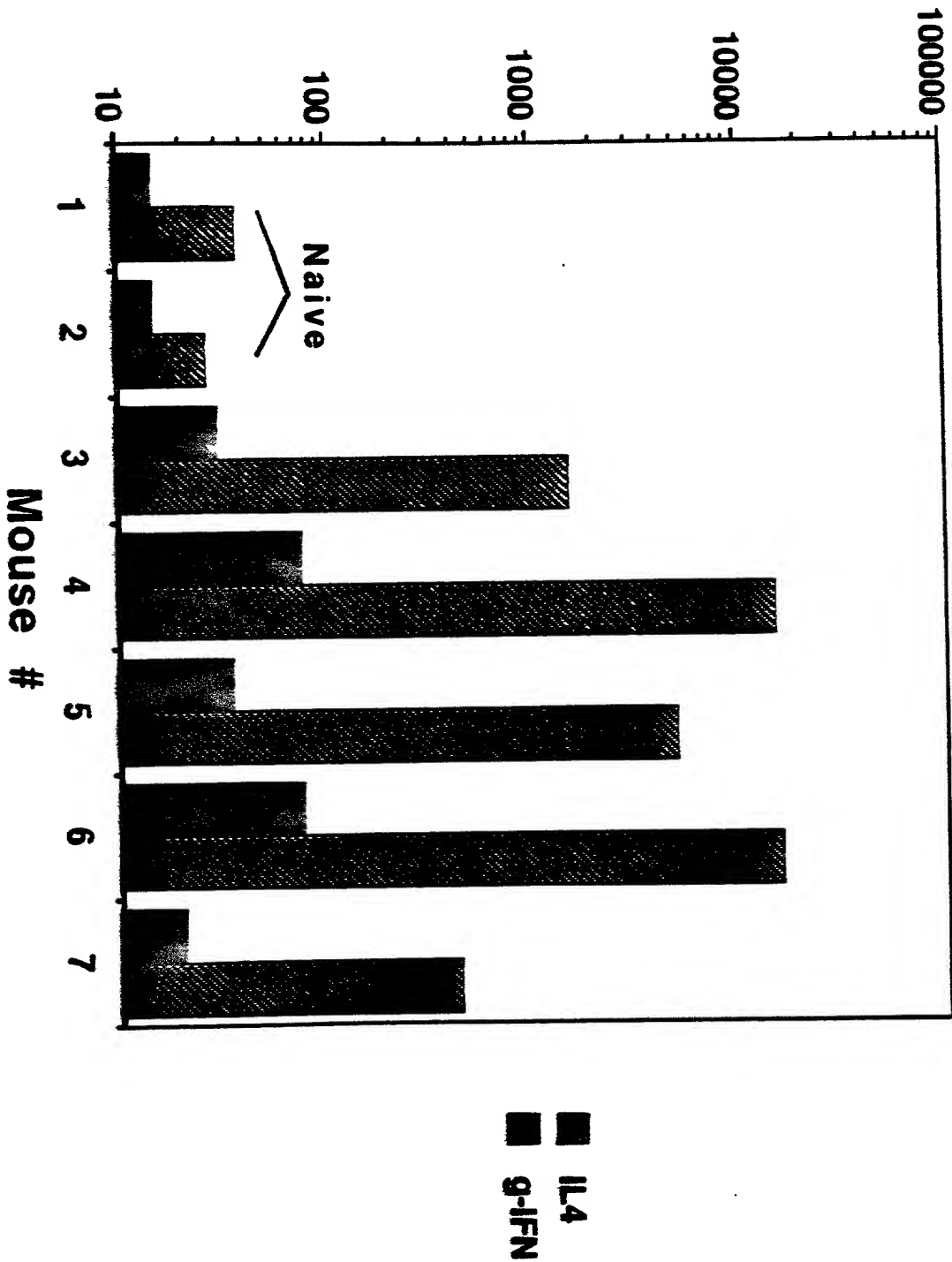
Anti-gp120 ELISA
titers are indicated
above each sample

Mouse #

FIGURE 14

Cytokine Concentration (pg/ml)

In Vitro Cytokine Secretion by tPA-gp120 Vaccines



-Using supernatants of splenic lymphocyte cultures (using 5 ug/ml rgp120) from gp120 PNV-vaccinated mice (1.6 ug, 2X)

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FIGURE 15

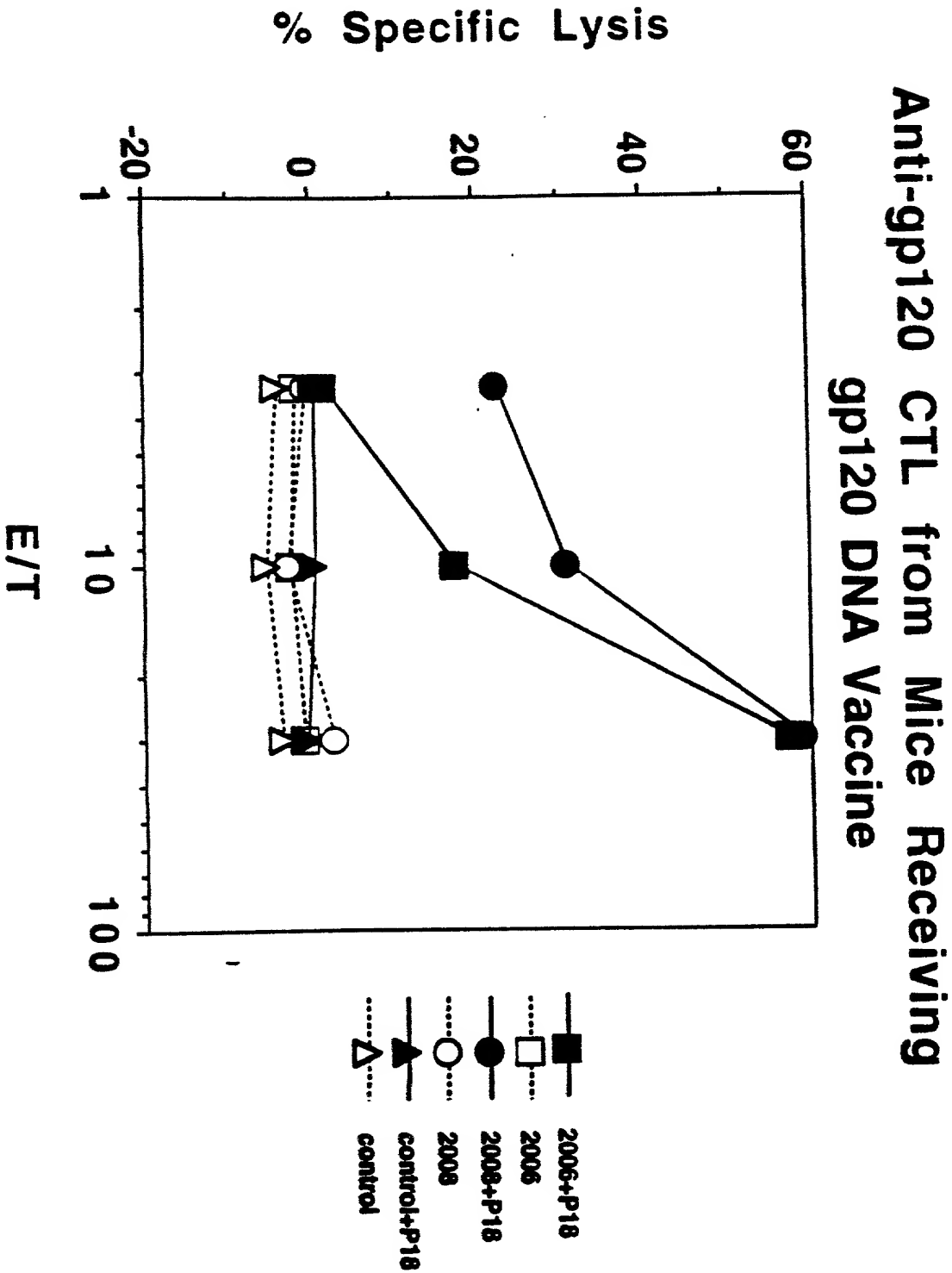
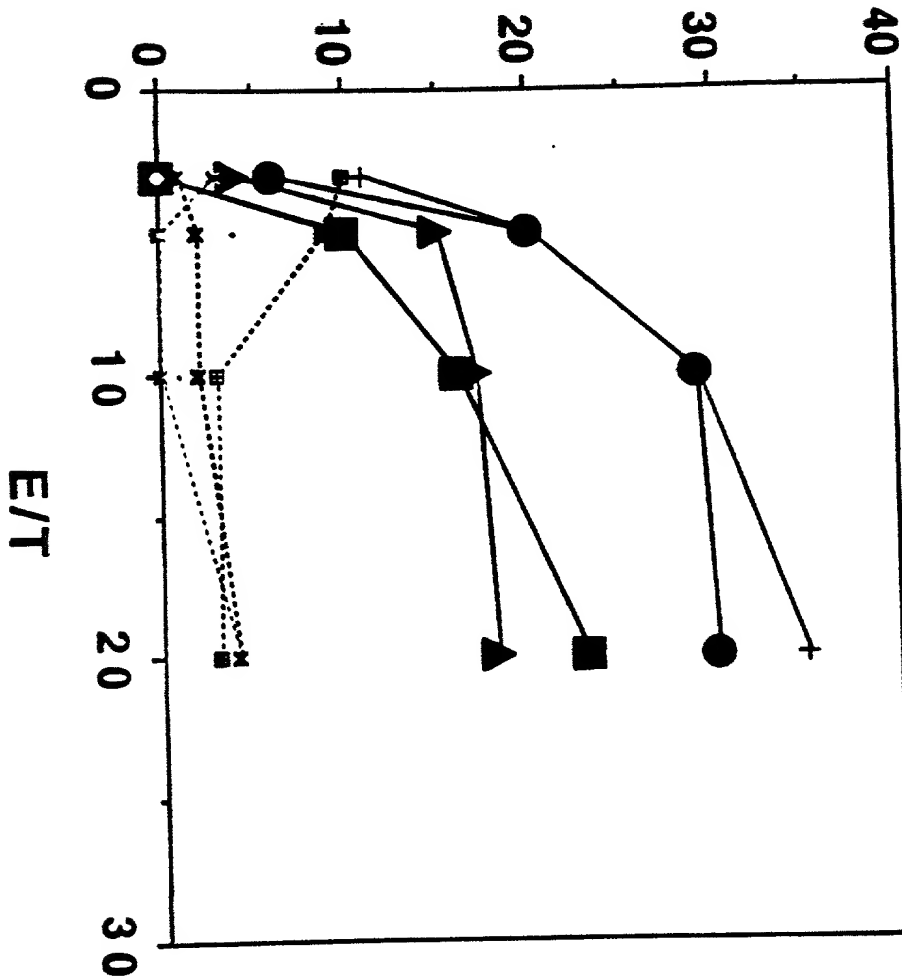


FIGURE 16

% Specific Lysis

Anti-gp120 CTL from Rhesus Monkeys
Receiving HIV env DNA Vaccine



● 92-0043/env
 ■ 92-0063/env
 ▲ 92-0071/env
 + 92-0019/env
 × 92-0079
 * 92-0088
 * 92-0069

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COORDINATE IN VIVO GENE EXPRESSION

the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application

Serial No 08/207,526

on 7-Mar-1994

and was amended

on (if applicable).

☒ was filed as PCT international application

Number PCT/US95/02633

on 3-Mar-1995

and was amended under PCT Article 19

on (if applicable).

☐ is being filed as PCT international application, Attorney Docket No.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Continued)
Reference to PCT International Applications

ATTORNEY'S DOCKET NUMBER
19188PI

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS			STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
08/207,526	7-Mar-1994				X
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
PCT/US95/02633	3-Mar-1995			X	

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

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Christine E. Carty
908-594-6734

201	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
202	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 <i>Margaret V. Liu</i>	SIGNATURE OF INVENTOR 202 <i>John W. Shiver</i>	SIGNATURE OF INVENTOR 203 <i>Helen C. Perry</i>
DATE 9/24/96	DATE 9/16/96	DATE 9/16/96

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): LIU ET AL.

Group Art Unit: 1805

Serial No.: 08/702,502

Examiner: NANCY J. DEGAN

Filed: March 3, 1997

For: COORDINATE IN VIVO EXPRESSION

ASSOCIATE POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In connection with the above-identified application the undersigned attorney and/or agent of record, hereby appoints J. MARK HAND Registration No. 36,545 c/o Patent Dept., MERCK & CO., INC., P.O. Box 2000, Rahway, New Jersey 07065-0907, an associate attorney and/or agent, to prosecute this application, to make alterations and amendments therein, to receive the patent and to transact all business in the Patent and Trademark Office connected therewith.

All communications in connection with the prosecution of the above-identified application should be sent to

J. MARK HAND c/o Patent Dept., MERCK & CO., INC., P.O. Box 2000, Rahway, New Jersey 07065-0907.

Respectfully submitted,

Signed at Rahway, New Jersey

this 13T day

of MAY 1998

By: Jack L. Tribble

Attorney I hereby for Applicant(s)

Reg. No. 32,633

(732)594- 5321

DATE: May 13, 1998